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(54) Title: SKIN CONDITIONING COMPOSITIONS CONTAINING COMPOUNDS FOR MIMICKING THE EFFECT ON SKIN OF RETINOIC ACID

(57) Abstract: A skin care product comprising from about 0.001 % to about 10 % of a retinoid, in combination with 0.0001 % to about 50 % of a combination of retinoid boosters.

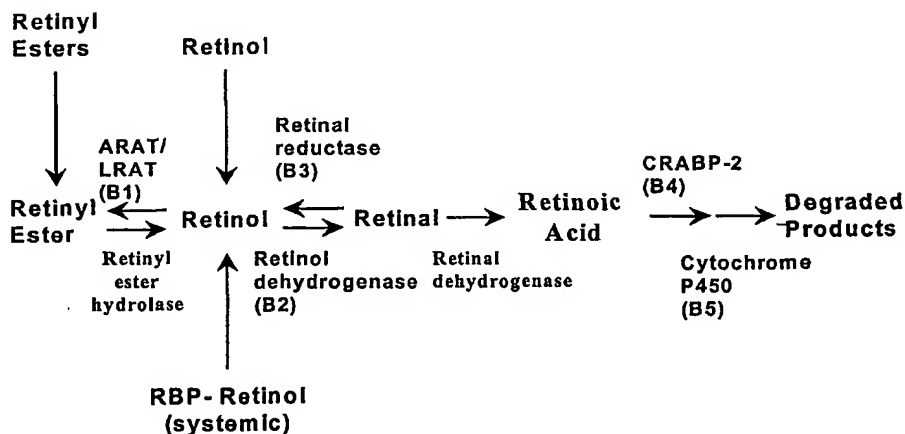
SKIN CONDITIONING COMPOSITIONS CONTAINING COMPOUNDS  
FOR MIMICKING THE EFFECT ON SKIN OF RETINOIC ACID

The present invention relates to cosmetic skin conditioning  
5 compositions containing certain compounds which mimic the  
effect on skin of retinoic acid.

Retinol (vitamin A) is an endogenous compound which occurs  
naturally in the human body, and is essential for normal  
10 epithelial cell differentiation. Natural and synthetic  
vitamin A derivatives have been used extensively in the  
treatment of a variety of skin disorders and have been used  
as skin repair or renewal agents. Retinoic acid has been  
employed to treat a variety of skin conditions, e.g., acne,  
15 wrinkles, psoriasis, age spots and discoloration. See e.g.,  
Vahlquist, A. et al., *J. Invest. Dermatol.*, Vol. 94, Holland  
D.B. and Cunliffe, W.J. (1990), pp. 496-498; Ellis, C.N. et  
al., "Pharmacology of Retinols in Skin", *Vasel, Karger*, Vol.  
3, (1989), pp. 249-252; Lowe, N.J. et al., "Pharmacology of  
20 Retinols in Skin", Vol. 3, (1989), pp. 240-248; PCT Patent  
Application No. WO 93/19743.

It is believed that retinol esters and retinol are  
enzymatically converted in the skin into retinoic acid  
25 according to the following mechanism:

## Retinol metabolism in the epidermis: enzyme names



The present invention is based on the discovery that certain compounds enhance the conversion of retinyl esters and retinol to retinoic acid. The compounds are collectively termed "boosters" and are coded as groups B1 to B5 according to the boosting mechanism of the particular compound. The mechanism of the booster groups is as follows: inhibiting ARAT/LRAT (AcylCoenzymeA(CoA): retinol acyl transferase/Lecithin: retinol acyl transferase) activity (B1), enhancing retinol dehydrogenase activity (B2), inhibiting retinal reductase activity (B3), antagonising CRABP-II (Cellular Retinoic Acid Binding Protein II) binding of retinoic acid (B4) and inhibiting cytochrome P450 dependent retinoic acid oxidation (B5).

The boosters alone or in combination with each other potentiate the action of retinoids by increasing the conversion of the retinoids to retinoic acid and preventing

the degradation of retinoic acid. The boosters act in conjunction with a retinoid (e.g. retinol, retinyl esters, retinal, retinoic acid), the latter being present endogenously in the skin. The preferred compositions, however, include a retinoid in the composition, co-present with a booster or a combination of boosters, to optimise performance.

Several patents by Granger et al describe the use of retinoid boosters in cosmetic compositions to improve the efficacy of retinol and retinyl esters (US patent numbers: 5759556, 5756109, 5747051, 5716627, 5811110, 5536740, 5747051, 5599548, 5955092, 5885595, 5759556, 5693330). The boosters described in these patents are restricted to class B1 and B5. Furthermore Johnson & Johnson have a series of patents which describe the use of molecules which fall into class 5 booster molecules (U.S. 5028628; U.S. 5037829; U.S. 5151421; U.S. 476852; U.S. 5500435; U.S. 5583136; U.S. 5612354).

20

The molecules which act as retinoid boosters are common ingredients in cosmetic products. There is considerable prior art describing their use in cosmetic compositions. There is substantial prior art describing the use of two or more of these molecules in the same composition. Some of the examples of the prior art are as in US 5,665,367, US 5747049, US 5853705, US 5766575, and US 5849310.

However, the prior art does not describe synergy arising from combinations of booster molecules. This observation of a synergistic boosting of retinoid activity from

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combinations of booster molecules was an unexpected finding. The prior art does not describe optimal concentrations or ratios of booster molecules or ratios of booster molecules to that of retinoids. Thus, the present invention is novel  
5 in that by combining cosmetic retinoids with booster molecules, at the most appropriate concentrations or ratios, a substantial improvement in efficacy of the retinoids is obtained.

10 The classes of boosters suitable for use in the present invention include but are not limited to the boosters listed in Tables B1 through to B5.

#### Best Groups of Boosters

15

##### **B1 Compounds**

1. Fatty Acid Amides	These are readily commercially available and have the added advantage of being surfactants and thus help generate emulsions suitable for cosmetic preparations.
2. Ceramides	These can additionally act as precursors of stratum corneum barrier ceramides.
3. Carotenoids	These can offer some UV protection and act as natural colorants.
4. Flavanoids	Natural antioxidants.
5. Cyclic fragrances	These are readily commercially available and additionally can be used to fragrance the product.
6. Non-cyclic fragrances	These can be used to fragrance the product.
7. Phospholipid analogues	These can be utilised by skin cells to nourish the generation of barrier components.
8. Ureas	These are readily commercially available and can also act as preservatives for the product.

**B2 Compounds**

1. Phosphatidyl choline	Most preferred as most active activator of Retinol Dehydrogenase
2. Sphingomyelin	

5 **B3 Compounds**

Arachidonic Acid Linoleic Acid Linolenic Acid Myristic Acid	Fatty Acids which can be useful in maintaining stratum corneum barrier
Linoleic Acid Linolenic Acid	Essential Fatty Acids
Arachidonic Acid Myristic Acid	Non-essential fatty acids
Glycyrrhetic Acid	Polycyclic triterpene carboxylic acid which is readily obtained from plant sources.
Phosphatidyl ethanolamine	Can be incorporated into cellular membranes.

**B4 Compounds**

10

Hexadecanedioic acid 12-hydroxystearic acid Isostearic acid	Saturated fatty acids.
Linseed oil Elaidic acid	Unsaturated fatty acids
Elaidic acid Isostearic acid Hexadecanedioic acid	Solid at room temperature
Linseed oil 12-hydroxystearic acid	Liquid at room temperature

**B5 Compounds**

Bifonazole Climbazole Clotrimazole Econazole Ketoconazole Miconazole	Antimicrotics
Climbazole	Readily commercially available
Lauryl hydroxyethylimidazoline	Compounds which are readily commercially available and have the added advantage of being surfactants and thus help generate emulsions suitable for cosmetic preparations.
Quercetin	Naturally occurring flavanoid which has antioxidant properties.
Coumarin	Natural colorant
Quinolines Isoquinolines	
Metirapone	

5 The present invention includes, in part, a skin conditioning composition containing from about 0.0001% to about 50%, preferably from 0.001% to 10%, most preferably from 0.001% to 5% by weight of the composition of a booster or combination of boosters and a cosmetically acceptable vehicle.

10

The boosters or combinations thereof included in the inventive compositions are selected from the group consisting of:

- 15 (a) a booster selected from the group consisting of  
B2; B3; B4;
- (b) binary combinations of boosters selected from the group consisting of:

B1/B2; B1/B3; B1/B4; B1/B5; B2/B3, B2/B4; B2/B5,  
B3/B4; B3/B5; B4/B5

5 (c) ternary combinations of boosters selected from the  
group consisting of:

B1/B2/B3; B1/B2/B4; B1/B2/B5; B1/B3/B4; B1/B3/B5;  
B1/B4/B5; B2/B3/B4; B2/B3/B5; B2/B4/B5; B3/B4/B5

10 (d) quaternary combinations of boosters selected from  
the group consisting of:

B1/B2/B3/B4; B1/B2/B3/B5; B1/B2/B4/B5;  
B1/B3/B4/B5; B2/B3/B4/B5;

and

15 (e) a combination of five groups of boosters:  
B1/B2/B3/B4/B5.

The preferred compositions include from about 0.001% to about  
10%, by weight of the composition of a retinoid.

20

The compounds included in the present invention as boosters  
are selected based on the ability of such compounds to pass,  
at a certain concentration listed in Table A, in-vitro Assays  
for a specific enzymes as described below under sections 2.1  
25 through to 2.7. Such a booster is included in the present  
invention even if it is not explicitly mentioned herein. Put  
another way, if a compound inhibits or enhances sufficiently  
an enzyme in an assay described below, it will act in  
combination with a retinoid to mimic the effect on  
30 keratinocytes (skin cells) of retinoic acid, and thus it is  
included within the scope of the present invention.

The term "conditioning" as used herein means prevention and treatment of dry skin, acne, photo-damaged skin, appearance of wrinkles, age spots, aged skin, increasing stratum corneum flexibility, lightening skin colour, controlling sebum excretion and generally increasing the quality of skin. The composition may be used to improve skin desquamation and epidermal differentiation.

- 10 The presence of the selected compounds in the inventive product substantially improves the performance of a retinoid.

The inventive compositions contain, as a preferred ingredient, a retinoid, which is selected from retinyl esters, retinol, retinal and retinoic acid, preferably retinol or retinyl ester. The term "retinol" includes the following isomers of retinol: all-trans-retinol, 13-cis-retinol, 11-cis-retinol, 9-cis-retinol, 3,4-didehydro-retinol, 3,4-didehydro-13-cis-retinol; 3,4-didehydro-11-cis-retinol; 3,4-didehydro-9-cis-retinol. Preferred isomers are all-trans-retinol, 13-cis-retinol, 3,4-didehydro-retinol, 9-cis-retinol. Most preferred is all-trans-retinol, due to its wide commercial availability.

- 25 Retinyl ester is an ester of retinol. The term "retinol" has been defined above. Retinyl esters suitable for use in the present invention are C<sub>1</sub>-C<sub>30</sub> esters of retinol, preferably C<sub>2</sub>-C<sub>20</sub> esters, and most preferably C<sub>2</sub>, C<sub>3</sub>, and C<sub>16</sub> esters because they are more commonly available. Examples of retinyl esters include but are not limited to: retinyl palmitate, retinyl
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formate, retinyl acetate, retinyl propionate, retinyl butyrate, retinyl valerate, retinyl isovalerate, retinyl hexanoate, retinyl heptanoate, retinyl octanoate, retinyl nonanoate, retinyl decanoate, retinyl undecanoate, retinyl laurate, retinyl tridecanoate, retinyl myristate, retinyl pentadecanoate, retinyl heptadecanoate, retinyl stearate, retinyl isostearate, retinyl nonadecanoate, retinyl arachidonate, retinyl behenate, retinyl linoleate, and retinyl oleate.

10

The preferred ester for use in the present invention is selected from retinyl palmitate, retinyl acetate and retinyl propionate, because these are the most commercially available and therefore the cheapest. Retinyl linoleate and retinyl oleate are also preferred due to their efficacy.

15

Retinol or retinyl ester is employed in the inventive composition in an amount of from about 0.001% to about 10%, preferably in an amount of from about 0.01% to about 1%, most preferably in an amount of from about 0.01% to about 0.5%.

20

The essential ingredient of the inventive compositions is a compound which passes in vitro Assays described below in sections 2.1 through to 2.7. A compound suitable for use in the present invention inhibits or enhances at a concentration listed in Table A an enzyme to at least a broad % listed in Table A.

25

## Section A: Identification of Booster:

**TABLE A**  
**Booster Test Concentrations and % Inhibition/Increase**

5

ARAT / LRAT Assay (To identify B1 boosters)		
Invention	Compound Concentration	% Inhibition
Broad	100 $\mu$ M	> 10%
Preferred	100 $\mu$ M	> 25%
Most Preferred	100 $\mu$ M	> 40%
Optimum	100 $\mu$ M	> 50%

Retinol Dehydrogenase Assay (To identify B2 boosters)		
Invention	Compound Concentration	% Increase
Broad	100 $\mu$ M	> 10%
Preferred	100 $\mu$ M	> 15%
Most Preferred	100 $\mu$ M	> 20%
Optimum	100 $\mu$ M	> 25%

10

Retinal Reductase Assay (To identify B3 boosters)		
Invention	Compound Concentration	% Inhibition
Broad	100 $\mu$ M	> 5%
Preferred	100 $\mu$ M	> 10%
Most Preferred	100 $\mu$ M	> 20%
Optimum	100 $\mu$ M	> 35%

CRABPII Antagonist Assay (To identify B4 boosters)		
Invention	Compound : Retinoic acid Ratio	% Inhibition
Broad	7000 : 1	> 25%
Preferred	7000 : 1	> 50%
Most Preferred	70 : 1	> 25%
Optimum	70 : 1	> 50%

Retinoic Acid Oxidation Assay (To identify B5 boosters)

Invention	Compound Concentration	% Inhibition
Broad	100 $\mu$ M	> 25%
Preferred	100 $\mu$ M	> 45%
Most Preferred	100 $\mu$ M	> 70%
Optimum	100 $\mu$ M	> 80%

The in vitro Microsomal Assays employed for determining the suitability of the inclusion of the compound in the inventive compositions are as follows:

#### 1. Materials

All-trans-retinol, all-trans-retinoic acid, palmitoyl-CoA, dilauroyl phosphatidyl choline, NAD, and NADPH were purchased from Sigma Chemical Company. Stock solutions of retinoids for the microsomal assays were made up in HPLC grade acetonitrile. All retinoid standard stock solutions for HPLC analysis were prepared in ethanol, stored under atmosphere of  $N_2$  at  $-70^\circ C$  and maintained on ice under amber lighting when out of storage. Other chemicals and the inhibitors were commercially available from cosmetic material suppliers or chemical companies such as Aldrich or International Flavours and Fragrances.

#### 2. Methods

##### 2.1 Isolation of RPE microsomes (modified from (1))

50 frozen hemisected bovine eyecups, with the retina and aqueous humor removed were obtained from W. L. Lawson Co.,

Lincoln, NE, USA. The eyes were thawed overnight and the colored iridescent membrane was removed by peeling with forceps. Each eyecup was washed with 2x 0.5mL cold buffer (0.1M PO<sub>4</sub> / 1mM DTT / 0.25M sucrose, pH 7) by rubbing the  
5 darkly pigmented cells with an artist's brush or a rubber policeman. The cell suspension was added to the iridescent membranes and the suspension was stirred for several minutes in a beaker with a Teflon stir bar. The suspension was filtered through a coarse filter (Spectra/Por 925µ pore size  
10 polyethylene mesh) to remove large particles, and the resulting darkly colored suspension was homogenized using a Glas-Col with a motor driven Teflon homogenizer.

The cell homogenate was centrifuged for 30 min. at 20,000g  
15 (Sorvaal model RC-5B centrifuge with an SS34 rotor in 2.5x10cm tubes at 14,000 RPM). The resulting supernatant was subjected to further centrifugation for 60 min. at 150,000g (Beckman model L80 Ultracentrifuge with an SW50.1 rotor in 13x51mm tubes at 40,000 RPM). The resulting pellets were  
20 dispersed into ~5mL 0.1M PO<sub>4</sub> / 5mM DTT, pH 7 buffer using a Heat Systems Ultrasonics, Inc. model W185D Sonifier Cell Disruptor, and the resulting microsomal dispersion was aliquoted into small tubes and stored at -70°C. The protein concentrations of the microsomes were determined using the  
25 BioRad Dye binding assay, using BSA as a standard.

## 2.2 Isolation of rat liver microsomes (4)

Approximately 6 grams of frozen rat liver (obtained from  
30 Harlan Sprague Dawley rats from Accurate Chemical and Scientific Corp.) was homogenized in 3 volumes of 0.1M tris /

0.1M KCl / 1mM EDTA / 0.25M sucrose, pH 7.4 buffer using a Brinkmann Polytron. The resulting tissue suspension was further homogenized in the motor driven Teflon homogenizer described above. The resulting homogenate was successively  
5 centrifuged for 30 min. at 10,000g, 30 min. at 20,000g, and 15 min. at 30,000g, and the resulting supernatant was ultra-centrifuged for 80 min. at 105,000g. The pellet was sonicated in ~5mL of 0.1M PO<sub>4</sub> / 0.1mM EDTA / 5mM MgCl<sub>2</sub>, pH 7.4 buffer as described above and stored as aliquots at -70°C.  
10 Protein concentrations were determined as described above.

### 2.3 Assay for ARAT and LRAT activity (To identify B1)

The procedure below was a modification of a method described  
15 in the literature (2). The following buffer was prepared and stored at 4°C: 0.1M PO<sub>4</sub> / 5mM dithiothreitol, pH 7.0 (PO<sub>4</sub>/DTT). On the day of the assay, 2mg BSA per mL of buffer was added to give a PO<sub>4</sub> / DTT / BSA working buffer. 1mM retinol substrate was prepared in acetonitrile and stored in  
20 amber bottles under nitrogen gas at -20°C. Solutions of 4mM Palmitoyl-CoA in working buffer (stored in aliquots) and 4mM dilauroyl phosphatidyl choline in ethanol were prepared and stored at -20°C. Inhibitors were prepared as 10mM stock solutions in H<sub>2</sub>O, ethanol, acetonitrile or DMSO. The quench  
25 solution was prepared using pure ethanol containing 50µg/mL butylated hydroxytoluene (BHT), and a hexane solution containing 50µg/mL BHT was used for the extractions.

To a 2 dram glass vial, the following were added in order: PO<sub>4</sub>  
30 / DTT / BSA buffer to give a total volume of 500µL, 5µL acyl donor (4mM palmitoyl-CoA and/or dilauroyl phosphatidyl

choline), 5 $\mu$ L inhibitor or solvent blank (10mM stock or further dilutions) followed by approximately 15 $\mu$ g of RPE microsomal protein (approximately 15 $\mu$ L of a ~1mg/mL microsomal protein aliquot). The mixture was incubated for 5 min. at 37°C to equilibrate the reaction temperature and then 5 $\mu$ L 1mM retinol was added. The vials were capped, vortexed for 5 seconds and incubated for 30-90 minutes at 37°C. The reaction was quenched by adding 0.5mL ethanol/BHT. The retinoids were extracted by adding 3mL hexane/BHT, vortexing the tubes for several seconds several times and centrifuging the tubes at low speed for 5 min. to quickly separate the layers. The upper hexane layer was removed into a clean vial, and the aqueous layer re-extracted with another 3mL hexane/BHT, as described above. The hexane layers were combined, and the hexane evaporated by drying at 37°C under a stream of nitrogen gas on a heated aluminum block. The dried residue was stored at -20°C until HPLC analysis. The amount of retinyl palmitate and retinyl laurate was quantitated for ARAT and LRAT activity, respectively, by integration of the HPLC signal as described below.

Note that the incubation solution contains 40 $\mu$ M acyl donor, 100 $\mu$ M or less inhibitor, 10 $\mu$ M retinol, approximately 30 $\mu$ g/mL microsomal protein, and nearly 0.1M PO<sub>4</sub>/ pH 7 / 5mM DTT / 2mg/mL BSA. All steps subsequent to the addition of retinol were done in the dark or under amber lights.

#### 2.4 Assay for Retinol Dehydrogenase Activity (To identify B2)

The following stock solutions were prepared:

- 50mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4 buffer, sterile filtered.  
10mM all trans Retinol (Sigma R7632) in DMSO.  
200mM Nicotinamide adenine dinucleotide phosphate, sodium salt (NADP) (Sigma N0505) in sterile water.
- 5 40mM test compound in appropriate solvent (water, buffer, ethanol, chloroform or DMSO).  
1:10 dilution of rat liver Microsomes in 50mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4 buffer (4µg/µl).
- 10 In a two-dram glass vial with screw cap, the following were added in order:
- Buffer to give a final volume of 400µl  
25µl diluted Microsomes (final = 100µg) - boiled Microsomes  
15 were used for controls and regular Microsomes for test samples.  
4µl of 200mM NADP (final = 2mM)  
1µl of 40mM test compound (final = 100µM)  
8µl of 10mM retinol (final = 200µM)
- 20 The vials were incubated in a 37°C shaking water bath for 45 minutes. 500µl ice-cold ethanol was added to each vial to quench the reaction. The retinoids were extracted twice with ice cold hexane (2.7ml per extraction). Retinyl acetate (5µl  
25 of a 900µM stock) was added to each vial during the first extraction as a means of monitoring the extraction efficiency in each sample. Samples were vortexed for ten seconds before gently centrifuging for five minutes at 1000rpm, 5°C in a Beckman GS-6R centrifuge. The top hexane layer containing  
30 the retinoids was removed from the aqueous layer after each extraction to a clean two-dram vial. The hexane was

evaporated off under a gentle stream of nitrogen gas. The dried residue was then stored at -20°C until HPLC analysis.

### 2.5 Assay for Retinal Reductase Activity (To identify B3)

5

All stock solution were prepared as above with the following substitutions:

10 10mM all trans Retinaldehyde (Sigma R2500) in DMSO - instead of retinol.

200mM, Nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt (NADPH) (Sigma N7505) in sterile water - instead of NADP.

15 In a two-dram glass vial with screw cap, add the following in order:

Buffer to give a final volume of 400µl

25µl diluted Microsomes (final = 100µg) - use boiled  
20 Microsomes for controls and regular Microsomes for test samples.

4µl of 200mM NADPH (final = 2mM)

1µl of 40mM test compound (final = 100µM)

3µl of 10mM retinaldehyde (final = 75µM)

25

Follow the same incubation and extraction procedure as detailed above.

## 2.6 Assay for CRABPII antagonists (To identify B4)

### 2.6.1. Synthesis of CRABPII

#### a. System of expression

5 The gene CRABPII was cloned in pET 29a-c(+) plasmid (Novagen). The cloned gene was under control of strong bacteriophage T7 transcription and translation signals. The source of T7 polymerase was provided by the host cell E.coli BLR(DE3)pLysS (Novagen). The latter has a chromosomal copy  
10 of T7 polymerase under lacUV5 control, induced by the presence of IPTG.

The plasmid was transferred into E. coli BLR(DE3)pLysS cells by transformation according to the manufacturer protocol  
15 (Novagen).

#### b. Induction

An overnight culture of the transformed cells was diluted 1:100 into 2xYT containing 50 µg/mL kanamycin and 25µg/mL  
20 chloramphenicol. The cells grew while shaking at 37°C until the OD at 600 nm reached 0.6-0.8. Then IPTG was added to a final concentration of 1mM and the culture was incubated for an additional two hours. The cells were harvested by centrifugation at 5,000g for 10 minutes at room temperature.  
25 The pellet was stored at -20°C.

### 2.6.2. Purification

Purification was performed according to the method described in Norris and Li, 1997.

#### 30 a. Lysis

The frozen pellet was thawed at RT and resuspended in 1-2 pellet volumes of freshly prepared lysis buffer (50 mM Tris-HCl, pH 8, 10%(w/v) sucrose, 1 mM EDTA, 0.05%(w/v) sodium azide, 0.5 mM DTT, 10 mM MnCl<sub>2</sub>, 2.5 mM phenylmethylsulfonyl fluoride, 2.5 mM benzamidine, 6µg/mL DNase). The lysate was incubated for 30 mins. at room temperature. Further lysis was accomplished by sonication (six 30-sec bursts at 10,000 psi alternated with five 30-sec delay on ice). The insoluble fraction of the lysate was removed by centrifugation at 10 15,000 rpm 1 hour at 4°C and the supernatant is stored at -20°C.

b. Gel filtration on Sephacryl S300

The supernatant from step a. was loaded onto a 2.5x100 cm column of sephacryl S-300 (Pharmacia) at room temperature. 15 The elution buffer was 20 mM Tris-HCl, pH 8, 0.5mM DTT, 0.05% sodium azide (buffer A). The flow rate was 2mL/min. Collected 2-mL fractions were checked for ultraviolet absorbance at 280 nm. The fractions representing the peaks 20 were examined by SDS-page for the presence of CRABPII.

c. Anion-exchange chromatography.

2 mL of gel filtration fractions containing CRABPII were loaded onto a quaternary amine anion-exchange column FPLC 25 (Fast Protein Liquid Chromatography) type monoQ (Pharmacia). CRABPII was eluted using a gradient buffer from 100% buffer A to 30% buffer B (100 % buffer B = buffer A + 250 mM NaCl) over a 20-min period at room temperature. 1 mL-fractions were collected every minute. Once more, the presence of 30 CRABPII was checked by SDS page. CRABPII was stored at 4°C before freeze-drying using a Micromodulyo 1.5K with vial.

platform attachment (Edwards High Vacuum International). The desiccated samples were stored at room temperature until their use in the binding assay.

5 d. Detection of the presence of CRABPII

The expression and purification of CRABPII was validated using denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis on a 7-15% polyacrylamide gel (Biorad). 10  $\mu$ L samples were mixed with 10  $\mu$ L of 2X loading buffer (100 mM  
10 Tris-HCl pH6.8, 4% SDS, 0.2% BPB, 20% glycerol, 1mM DTT) and denatured by heating (2 mins. at 80°C). The samples were loaded onto the gel that was immersed in a 1X Tris-glycine buffer (Biorad) and a constant current (25 mA) was applied for 1 hour at room temperature. After Coomassie blue  
15 staining, the protein was identified according to its molecular weight as determined with the Benchmark pre-stained protein ladder (Gibco BRL).

A western blot was used to confirm the presence of CRABPII.  
20 The proteins separated on the SDS-PAGE were transferred on an Immobilon-P transfer membrane (Millipore) using a Biorad cassette. The transfer occurred in 1X Tris-glycine buffer (Biorad) + 10% methanol. An electrical current (60 mA) was applied for 3 hours to allow the protein to migrate through  
25 the membrane. Afterwards, the membrane was blocked with 5% dry milk in 1X TBS for one hour at room temperature and probed with primary antibodies to CRABPII (1/1000 dilution of mouse anticolon 5-CRA-B3) in the same buffer at 4°C overnight. The following day, the membrane was washed with  
30 PBS (3 x 5 minutes) and then incubated with 1:2000 dilution of the secondary antibody, peroxidase conjugated anti-mouse

antibody (ECLTM, Amersham), for 1 hour at room temperature. The membrane was washed with 1xPBS (3x5 minutes) and the protein was detected using ECL detection kit according to the manufacturer instruction (Amersham).

5

The concentration of purified CRABPII was determined using BSA kit (Pierce).

#### 2.6.3. Radioactive Binding assay

10 220 pmol of CRABPII was incubated in 20 mM Tris-HCl buffer pH 7.4 with 15 pmol of radioactive all trans retinoic acid (NEN) in a total volume of 70 $\mu$ L. For the competitive assay, another ligand in excess (6670:1, 670:1 or 70:1) was added to the mix. The reaction occurred for one hour at room  
15 temperature in the dark. In order to separate the unbound all-trans retinoic acid from the bound all-trans retinoic acid, a 6kD cut-off minichromatography column (Biorad) was used. The storage buffer was discarded using a Microplex manifold for according to the manufacturer instruction  
20 (Pharmacia). The samples were loaded onto the column and the separation occurred by gravity over a 30-min period. Retinoic acid ("RA") bound to CRABPII appeared in the filtrate while free RA remained in the column. The radioactivity of the filtrate was measured by scintillation  
25 counter.

#### 2.7 Assay for NADPH dependent retinoic acid oxidation (To identify B5)

30 The procedure below is a modification of a method described in the literature (4). The following assay buffer was

prepared and stored at 4°C: 0.1M PO<sub>4</sub> / 0.1mM EDTA / 5mM MgCl<sub>2</sub>, pH 7.4. On the day of the assay, a 60mM NADPH solution in buffer was prepared. Inhibitor stocks, acidified ethanol / BHT quench solution, and hexane / BHT were prepared as  
5 described above. A working 1mM retinoic acid solution was prepared by dilution of a 15mM stock (in DMSO) with ethanol.

To a 2 dram vial, the following were added in order: assay buffer to give a final volume of 500µL, 20µL 60mM NADPH, 5µL  
10 inhibitor or solvent blank, followed by approximately 2mg of rat liver microsomal protein.

The mixture was incubated for 5 mins. at 37°C, then 5µL working 1mM retinoic acid solution was added. Incubation was  
15 continued for 60mins. at 37°C - the vials were not capped, since the oxidation process required molecular O<sub>2</sub> in addition to NADPH. Quenching was carried out with acidified ethanol/BHT and extraction was carried out with hexane/BHT as described above. Quantitation of the quickly eluting polar  
20 retinoic acid metabolites (presumed to be 4-oxo retinoic acid) was carried out by integration of the HPLC signal as described below.

All steps subsequent to the addition of retinoic acid were  
25 done in the dark or under amber lights. The final incubation solution contained 2.4mM NADPH, 100µM or less inhibitor, 10µM retinoic acid, approximately 4mg/mL rat liver microsomal protein and nearly 0.1M PO<sub>4</sub> / 0.1mM EDTA / 5mM MgCl<sub>2</sub>.

30 HPLC analysis of individual retinoids

Samples for retinoid quantitation by HPLC were prepared by dissolving the residue in each vial with 100 $\mu$ L of methanol. The solution was transferred to a 150 $\mu$ L glass conical tube within a 1mL shell vial, capped tightly, and placed inside a  
5 Waters 715 Autosampler. Aliquots of 60 $\mu$ L were injected immediately and analysed for retinoid content.

The chromatography instrumentation consisted of a Waters 600 gradient controller/pump, a Waters 996 Photodiode Array  
10 detector and a Waters 474 Scanning Fluorescence detector. Two HPLC protocols were used for retinoid analysis. For the ARAT and LRAT assay, the separation of retinol and retinol esters was performed with a Waters 3.9x300mm C18 Novapak reverse-phase analytical column and Waters Sentry NovaPak C18  
15 guard column with an 80:20(v/v) methanol/THF isocratic mobile phase adjusted to a flow rate of 1mL/min. for 10 min. The eluate was monitored for absorbance at 325nm and fluorescence at 325ex/480em.

20 A shorter Waters 3.9x150mm C18 Novapak reverse-phase analytical column and Waters Sentry NovaPak C18 guard column were used to separate retinoid acids and alcohols for the retinol and retinoic acid oxidation assays utilising a modification of a gradient system described by Barua (5).  
25 This system consisted of a 20 mins. linear gradient from 68:32(v/v) methanol/ water containing 10mM ammonium acetate to 4:1(v/v) methanol:dichloromethane followed by a 5 mins. hold at a flow rate of 1mL/min. The column eluate was monitored from 300nm to 400nm.

These protocols were selected based on their ability to clearly resolve pertinent retinoid acids, alcohols, aldehydes, and/or esters for each assay and relative quickness of separation. Identification of individual  
5 retinoids by HPLC was based on an exact match of the retention time of unknown peaks with that of available authentic retinoid standards and UV spectra analysis (300-400nm) of unknown peaks against available authentic retinoids.

10

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The boosters suitable for use in the present invention  
30 include but are not limited to the boosters listed in Tables B<sub>1</sub> through to B<sub>5</sub> below. The table below gives the booster

class (B<sub>1</sub> - B<sub>5</sub>), the chemical name of the compound, and the results from the appropriate assays used to identify the booster (i.e. ARAT/LRAT for B<sub>1</sub>, retinol dehydrogenase for B<sub>2</sub>, retinaldehyde inhibition for B<sub>3</sub>, CRABP is binding for B<sub>4</sub> and  
5 retinoic acid oxidation inhibition for B<sub>5</sub>.

## ARAT/LRAT Inhibitors (B1)

Class	Compound	%Inhibition Overall TG (-ROH/RE)	Overall TG (IC 50)	%Inhibition ARAT (10µm)	%Inhibition ARAT (100µm)	%Inhibition LRAT (10µm)	%Inhibition LRAT (100µm)
Carotenoid	Croctetin		3.75E-05	15%	34%	0	15%
Fatty Acid & Other Surfactants	Acetyl Sphingosine		6.78E-06	19%+/-12	62%+/-11	10%+/-10	50%+/-18
Fatty Acid Amides & Other Surfactants	C13 Beta-Hydroxy Acid/ Amide	17%			28%		25%
Fatty Acid Amides & Other Surfactants	Castor Oil MEA		3.25E-05				
Fatty Acid Amides & Other Surfactants	Cocamidopropyl Betaine				25%		
Fatty Acid Amides & Other Surfactants	Coco Hydroxyethyl- imidazoline		2.84E-07		68%		65%
Fatty Acid Amides & Other Surfactants	Cocoamide-MEA (or Cocoyl Monoethanol- amide)	11%			13%		34%
Fatty Acid Amides & Other Surfactants	Glycerol-PCA-Oleate				41%+/-6		58%+/-2
Fatty Acid Amides & Other Surfactants	Hexanoamide				20%		
Fatty Acid Amides & Other Surfactants	Hexanoyl Sphingosine		9.99E-05		28%+/-4		37%+/-9
Fatty Acid Amides & Other Surfactants	Hydroxyethyl-2- Hydroxy-C12 Amide		3.29E-05		35%		35%
Fatty Acid Amides & Other Surfactants	Hydroxyethyl-2- Hydroxy-C16 Amide				25%		30%
Fatty Acid Amides & Other Surfactants	Lauroyl Sarcosine				20%		
Fatty Acid Amides & Other Surfactants	Lidocaine				12%		0
Fatty Acid Amides & Other Surfactants	Linoleamide-DEA (or Linoleoyl Diethanolamide)	59%		12%+/-3	43%+/-3	11%+/-9	51%+/-15
Fatty Acid Amides & Other Surfactants	Linoleamide-NEA (or Linoleoyl Monoethanol- amide)		1.61E-05	14%	35%	20%+/-8	35%
Fatty Acid Amides & Other Surfactants	Linoleamidopropyl Dimethylamine				69%+/-18		75%+/-4
Fatty Acid Amides & Other Surfactants	Melinamide				64%+/-15		43%+/-21
Fatty Acid Amides & Other Surfactants	Myristoyl Sarcosine				41%+/-14		11%+/-11

Other Surfactants	2.80E-05	47%
Fatty Acid Amides & Other Surfactants	Oleyl Betaine	47%
Fatty Acid Amides & Other Surfactants	Palmitamide-MEA	6%
Fatty Acid Amides & Other Surfactants	Stearylhydroxyamide	10%
Fatty Acid Amides & Other Surfactants	Utrecht-1	54%
Fatty Acid Amides & Other Surfactants	Utrecht-2	83%+/-9
Fatty Acid Amides & Other Surfactants	Naringenin	33%
Flavanoids	Allyl Alpha-Ionone	16%+/-14
Fragrances	Alpha-Damascone	67%+/-27
Fragrances	Alpha-Ionone	45%+/-27
Fragrances	Alpha-Methyl Ionone	67%
Fragrances	Alpha-Terpineol	26%
Fragrances	Beta-Damascone	84%
Fragrances	Brahanol	70%
Fragrances	Damascone	70%
Fragrances	Delta-Damascone	87%
Fragrances	Dihydro Alpha-Ionone	13%
Fragrances	Ethyl Saffranate	51%
Fragrances	Fenchyl Alcohol	12%
Fragrances	Gamma-Methyl Ionone	21%
Fragrances	Isobutyl Ionone	8%
Fragrances	Isocyclogeraniol	18%
Fragrances	Isodamascone	80%
Fragrances	Lyzal	76%
Fragrances	Santalone	23%
Fragrances	Santanol	15%
Fragrances	Timberol	34%
Fragrances	Tonalid	50%
Fragrances	Traseolide	41%
Miscellaneous	Coco Trimethyl-ammonium Cl-	27%
Miscellaneous	Urosolic Acid	21%
Noncyclic Fragrances	Citral	20%
	1.46E-06	28%



5

10

Retinol Dehydrogenase Activators (B2)		
Class	Compound	%Increase Retinol Dehydrogenase
Phospholipid	Phosphatidyl Choline	21% increase
Phospholipid	Sphingomyelin	26% increase

## Retinaldehyde Reductase Inhibitors (B3)

Class	Compound	Overall TG(IC 50)	% Inhibition Retinal Reductase
Aldehyde	Vanillin	9.70E-03	6%
Fatty Acid	Arachidic Acid		20%
Fatty Acid	Arachidonic Acid		49%
Fatty Acid	Linoleic Acid	1.63E-04	62%+/-2
Fatty Acid	Linolenic Acid	1.34E-04	54%+/-16
Fatty Acid	Myristic Acid	1.72E-05	26%
Miscellaneous	Amsacrine	6.26E-06	22%+/-8
Miscellaneous	Carbenoxolone	3.61E-07	26%+/-2
Miscellaneous	Glycyrrhetic Acid	8.64E-06	38%+/-1
Phospholipid	Phosphatidyl ethanolamine		37%

## CRABPII Antagonists (B4)

Class	Compound	Overall TG(IC 50)	% Inhibition CRABPII
Fatty Acid	Elaidic Acid	6.50E-05	>50%
Fatty Acid	Hexadecanedioic Acid	1.30E-04	>50%
Fatty Acid	12-Hydroxystearic Acid	2.91E-05	>50%
Fatty Acid	Isostearic Acid	6.88E-05	>50%
Fatty Acids	Linseed Oil		>50%

## Retinoic Acid Oxidation Inhibitors (B5)

Class	Compound	Overall TG(IC 50)	%Inhibition		% Inhibition	
			Retinoic Acid (10µM)	Retinoic Acid (100µM)	Retinoic Acid(100µM)	Retinoic Acid(100µM)
Imidazole	Bifonazole		89%		100%	
Imidazole	Climbazole	4.47E-06	80%		92%	
Imidazole	Clotrimazole		76%		85%	
Imidazole	Econazole		88%		100%	
Imidazole	Ketoconazole	1.85E-07	84%		84%	
Imidazole	Miconazole	2.78E-07	74%		86%	
Fatty Acid Amides & Other Surfactants	Lauryl Hydroxyethylimidazole	4.67E-07				
Fatty Acid Amides & Other Surfactants	Oleyl Hydroxyethylimidazole	3.02E-05	54%		80%	
Flavonoids	Quercetin	6.29E-05	40%		74%	
Coumarin	Coumarin					
Quinoline	(7H-Benzimidazo [2,1-a]Benz [de]-Isoquinolin-7-one	8.59E-07				
Quinoline	Hydroxyquinoline (Carbostyryl)	3.64E-04				
Quinoline	Metirapone (2-Methyl-1,2-di-3- Pyridyl-1-Propane)				47%	

### SECTION B. Effects Of Booster Combinations:

In order to assess the effect of combinations of booster  
5 molecules an assay is required which encompasses the effect  
of each of the five booster classes. A single enzyme assay  
is not suitable for this purpose, as it will be specific  
only for one class of booster molecule. An assay which  
reflects retinoid concentration in keratinocytes is  
10 necessary to relate the effects of single booster molecules  
with combination of booster molecules. For this reason, a  
transglutaminase (Tgase) assay was utilised. Tgases are  
calcium dependent enzymes that catalyse the formation of  
covalent cross-links in proteins. Several Tgase enzymes are  
15 membrane bound in keratinocytes which is important for  
epidermal cell maturation. This enzyme is inhibited by  
retinoic acid. The higher the concentration of retinoic  
acid, the greater the inhibition of Tgase expression. Hence  
Tgase is a good marker of both keratinocyte differentiation  
20 and of the retinoid effect on keratinocytes.

#### Transglutaminase as a marker of skin differentiation

During the process of terminal differentiation in the  
25 epidermis, a 15nm thick layer of protein, known as the  
cornified envelope (CE) is formed on the inner surface of the  
cell periphery. The CE is composed of numerous distinct  
proteins which have been cross-linked together by the  
formation of NE-( $\gamma$ -glutamyl) lysine isodipeptide bonds  
30 catalysed by the action of at least two different

transglutaminases (TGases) expressed in the epidermis. TGase I is expressed in abundance in the differentiated layers of the epidermis, especially the granular layer, but is absent in the undifferentiated basal epidermis. Thus TGase I is a useful marker of epidermal keratinocyte differentiation with high TGase I levels indicating a more differentiated state. An ELISA based TGase I assay, using a TGase I antibody, was used to assess the state of differentiation of the cultured keratinocytes in the examples that follow.

10

Keratinocytes (cultured as described above) were plated in 96 well plates at a density of 4,000-5,000 cells per well in 200µl media. After incubation for two to three days, or until cells are ~50% confluent, the media was changed to media containing test compounds (five replicates per test). The cells were cultured for a further 96 hours after which time the media was aspirated and the plates stored at -70°C. Plates were removed from the freezer, and the cells were washed twice with 200µl of 1xPBS. The cells were incubated for one hour at room temperature (R/T) with TBS/5% BSA (wash buffer, bovine serum albumin). Next the TGase primary antibody was added: 50µl of monoclonal anti-Tgase I Ab B.C. diluted 1:2000 in wash buffer. The primary antibody was incubated for 2 hours at 37°C and then rinsed 6x with wash buffer. Cells were then incubated with 50µl of secondary antibody (Fab fragment, peroxidase conjugated anti-mouse IgG obtaining from Amersham) diluted 1:4,000 in wash buffer for two hours at 37°C, then rinsed three times with wash buffer. Following the rinse with washing buffer, the cells were rinsed 3x with PBS. For colourimetric development, the cells

30

were incubated with 100µl substrate solution (4 mg o-phenylenediamine and 3.3 µl 30% H<sub>2</sub>O<sub>2</sub> in 10ml 0.1M citrate buffer pH 5.0) for exactly five minutes, R/T, in darkness (under aluminum foil). The reaction was stopped by the  
5 addition of 50µl 4N H<sub>2</sub>SO<sub>4</sub>. The absorbance of samples was read at 492nm in a 96 well plate UV spectrophotometer. Out of the five replicates, four were treated with both antibodies, the fifth one was use as a Tgase background control. Tgase levels were determined and expressed as percentage control.

10

Details of of Tgase assay:

Prior to initiating experiments, to determine the effects of combinations of booster molecules standard Tgase assay  
15 conditions were investigated. A fully validated Tgase assay was established as follows:

A. Reagents

20	Cells: Human Keratinocytes (P2 in T75 flasks; P3 in 96 well assay plates) Primary Antibody: TGm specific monoclonal Ab B.C1	Neonatal Human foreskin  Biogenesis (Cat# 5560-6006)
25	Secondary Ab: Peroxidase labeled antimouse Ig F(ab)2	Amersham (Cat # NA9310)
30	Substrate solution: For 10 ml phosphate citrate buffer 4.0 mg o-phenylenediamine 3.3 µl of 30% H <sub>2</sub> O <sub>2</sub>	Sigma P-7288 Sigma H-1909

B. Media/Buffers

- Keratinocyte Growth Media (KGM) Clonetics (Cat# 3111)
- 5 Phosphate Buffered Saline;  
Dulbecco's without Ca/MgCl<sub>2</sub>) Life Technology  
(Cat # 14200-075 )
- Tris Buffered Saline
- 10 Blocking buffer (1xTBS + 5%  
dry milk) BioRad (Cat #170-6404)
- Washing buffer (1% dry milk in  
TBS + 0.05% Tween 20) Sigma (Cat # P-7949)
- 15 Phosphate citrate buffer: 1:1  
mixture of 0.2M dibasic  
sodium phosphate and 0.1 M  
citric acid Sigma (Cat # S-9763)  
Sigma (Cat # C-1909)
- 20 4 N H<sub>2</sub>SO<sub>4</sub>

C. Culture ware

- 25 96-well polypropylene microtitre  
plate Costar (Cat # 3595)
- 96-well polypropylene U-bottom  
plate Costar (Cat # 3794)
- 30 T75- vent cap Costar (Cat # 3376)

D: Instrumentation/Equipment

- 35 Biotek Model EL 340 Microplate  
reader Bio-tek Instruments Inc.  
Multiprobe II Packard

E: Cell Culture ProcedureSeeding of Keratinocytes in 96 well plates

- 5 1. A suspension of keratinocytes was prepared at a concentration of 3000 cells/200  $\mu$ l/ well in KGM medium (Used  $3 \times 10^5$  cells /12 ml media in each microtitre plate)
2. 200 $\mu$ l of the keratinocyte suspension was transferred into each of the inner 60 wells only.
- 10 3. 200 $\mu$ l of KGM media was pipetted into the outer wells (to maintain thermal equilibrium).
4. Each plate was incubated at 37°C and 5% CO<sub>2</sub> for 3 days or until cells are ~50% confluent.

15 Treatment of keratinocytes with samples.

5. Stock solutions of the samples were prepared in DMSO.
6. The samples were diluted to desired concentration with the final assay concentration of DMSO being 0.1 %.
- 20 7. 20  $\mu$ l of the sample was transferred into wells and 180  $\mu$ l of KGM medium added to give a final assay volume of 200  $\mu$ l.
8. Plates were incubated at 37°C and 5% CO<sub>2</sub> for 72 hours.
9. Media were completely removed from each well.
- 25 10. Wells were rinsed with 2x with 200  $\mu$ l of 1xPBS
11. Finally they were frozen for at least 1.5 hours at -70°C.

## F: Transglutaminase Assay

## 1. Block:

Incubate plates at room temperature with 200 µl/well of  
5 blocking buffer for 1 hour.

## 2. Primary Antibody:

Aspirate blocking buffer. Incubated with 100 µl/well  
of TGM-specific monoclonal antibody B.C1 (diluted  
1:2000 in washing buffer) at 37°C for at least 2 hours.  
10 The primary antibody was not added in background  
control wells.

## 3. Rinsed wells 6x with washing buffer.

## 4. Secondary Antibody:

Incubated with 100 µl/well peroxidase labeled anti-  
15 mouse IgF(ab)2 fragment (diluted 1:4000 in washing  
buffer) at 37°C for 2 hours.

5. Rinsed wells 3X with washing buffer (added 200µl) and  
aspirated after each rinse.

6. Rinsed wells 3X with PBS w/o Tween.

20 7. Incubated with 100 µl/well substrate solution at room  
temperature for exactly 5 minutes.

8. Stopped reaction with 50 µl/well 4N H<sub>2</sub>SO<sub>4</sub>.

9. Read absorbance at 492 nm in the Bio-tek plate reader.

## 25 I. Optimization Studies

a. Time Course of Transglutaminase Production

A time course experiment was conducted to determine the  
30 optimal incubation time for transglutaminase production  
in keratinocytes grown in 96-well plates (4000

cells/well). This time course study was conducted with multiple variables including dose response analyses of retinoic acid and retinol as well as incubation in the presence of 1.2 mM  $\text{CaCl}_2$ . Although the transglutaminase production in the control cells (0.1% DMSO) was not altered, both retinoic acid and retinol exhibited a dose dependent inhibition of transglutaminase production over the five day incubation period. The most pronounced retinoid effect was observed on day 2 and day 3. The maximal inhibition was observed on day 2 with the transglutaminase production being inhibited by 85% and 55% in the presence of the highest concentration (1  $\mu\text{M}$ ) of retinoic acid and retinol respectively. The same experiment was also conducted with varying cell density (3000 cells/well and 5000 cells/well) and comparable results were observed.

B: DMSO Sensitivity

Various concentrations of DMSO ranging from 0-2% were tested for the effect on transglutaminase production in keratinocytes. The assay was sensitive to DMSO concentration with significant inhibition of activity, above 0.5% DMSO. Hence, a final assay concentration of 0.1% was selected for subsequent sample concentration studies.

C: Dose Response Curves: Retinoic Acid and Retinol

Based on the data, day 3 was selected as the optimal time and 0.1%DMSO was selected as the concentration to be used for further testing. An additional dose

response experiment was carried out with retinoic acid and retinol in the presence of 0.1% DMSO, with the transglutaminase production being assayed on day 3. A good dose response was observed for Tgase inhibition by retinoic acid and retinol. 10<sup>-7</sup>M retinol gave an inhibition of Tgase in the linear range of concentration. Therefore, this concentration of retinol was chosen to evaluate the booster combinations.

D: Final conditions used to test boosters or combination of boosters

Days of incubation of keratinocytes with retinol and boosters	-	3 days
Final DMSO concentration	-	less than 0.1%
Retinol concentration	-	10 <sup>-7</sup> M (0.1µM)
Booster concentrations	-	10 mM to 0.1 nM

Using the above conditions, dose response for all the different boosters (B1-B5) were tested to identify the best concentration of booster to test in combinations.

Transglutaminase levels were determined and expressed in the Tables B1 through B5 either as:

(i) % (booster + retinol inhibition / control inhibition) - % (ROH inhibition / control inhibition), which measures the added effect of booster + retinol induced TGase inhibition over retinol alone, or

(ii) as an IC<sub>50</sub> value when the inhibitory effect of multiple booster concentrations was examined - this provides the concentration of booster which, in combination with a

constant retinol concentration of  $10^{-7}$  M, inhibits TGase by 50%.

Booster combinations and booster ratios:

5

It has been discovered surprisingly that certain compounds increase the endogenous levels of retinoic acid formation from retinol or retinyl esters by different mechanisms. These compounds are collectively called here as "retinoid  
10 boosters". These include: inhibitors of ARAT/LRAT (B1 boosters), inhibitors of retinaldehyde reductase (B3 boosters), inhibitors of retinoic acid binding to CRABP-2 (B4 boosters) and inhibitors of retinoic acid oxidation catalysed by cytochrome P450 enzymes (B5 boosters), or  
15 certain other compounds which enhance or activate retinol dehydrogenase (B2 boosters). These boosters are coded as groups B1 through to B5, as seen in chart 1 herein above.

The boosters alone or in combination with each other,  
20 potentiate the action of a retinoid by increasing the amount of retinol available for conversion to retinoic acid and inhibiting the degradation of retinoic acid. The boosters act in conjunction with a retinoid (e.g. retinol, retinyl ester, retinal, retinoic acid) the latter being present  
25 endogenously in the skin. The preferred compositions, however, include a retinoid in the composition, co-present with a booster, to optimise performance.

The present invention includes, in part, a second  
30 composition containing from about 0.0001% to about 50%,

preferably from 0.001% to 10%, most preferably from 0.001% to 5% by weight of the composition of at least one booster compound, or a combination of binary, tertiary, quaternary or 5 booster combinations. The combined concentration of the booster combinations of 0.001% to 5% in specified ratios as shown below, inhibit transglutaminase in an in vitro transglutaminase assay to more than 50%, and a cosmetically acceptable vehicle.

- 10 The boosters included in the inventive compositions are selected from the group consisting of:
- a. Two boosters, wherein both are selected from the group consisting of B2, B3 and B4;
  - b. Binary combinations of boosters selected from the group  
15 consisting of B1/B2; B1/B3, B1/B4; B1/B5; B2/B3, B2/B4; B2/B5; B3/B4, B3/B5; B4/B5
  - c. Ternary combinations of boosters selected from the group consisting of B1/B2/B3; B1/B2/B4; B1/B2/B5; B1/B3/B4; B1/B3/B5; B1/B4/B5; B2/B3/B4; B2/B3/B5;  
20 B2/B4/B5; B3/B4/B5
  - d. Quaternary combinations of boosters selected from the group consisting of B1/B2/B3/B4; B1/B2/B3/B5; B1/B2/B4/B5; B1/B3/B4/B5; B2/B3/B4/B5; and
  - e. A combination of five groups of boosters B1/B2/B3/B4/B5.

25

Booster to booster ratios:

The boosters of different classes (B1 to B5) in combinations  
30 as shown above have an optimal concentration of between 0.001% to 5% in a cosmetic product at specific ratios as

shown below for inhibition of Tgase activity to at least below 50%:

Invention	Ratios of boosters to boosters	Concentrations
5 Broad	1: 10,000 to 10,000:1	100 mM to 1 nM
Preferred	1: 1000 to 1000:1	10 mM to 10 nM
Most preferred	1:100 to 100:1	1 mM to 100 nM
10 Optimum	1:10 to 10:1	0.1 mM to 1 $\mu$ M

#### Retinoid to booster ratios:

The preferred composition includes a retinoid (e.g. retinol, retinyl ester, and retinaldehyde) in the composition, co-  
 15 present with a booster or a combination of the boosters, to optimise performance.

For optimum performance, the concentration of retinoid to booster should be present in the composition in ratios as  
 20 given below:

Invention	Ratios of boosters to retinoids	Concentrations
25 Broad	10,000:1 to 1:10,000	100 mM- 1 nM booster; 0.001-10% retinoids
Preferred	1000:1 to 1:1000	10 mM-10 nM booster; 0.001-10% retinoid
Most preferred	100:1 to 1:100	1 mM-100 nM booster; 0.01-1% retinoid
30		

#### Concentrations of individual boosters used in the examples:

35 Since the objective is to establish synergistic inhibition of transglutaminase expression by combinations of the active compounds with retinol, it was essential to determine the dose response profiles (IC<sub>20</sub> and IC<sub>50</sub> values) of the active compounds, when tested individually in the presence of

retinol. The detailed dose response of boosters belonging to B2-B4 is given in the tables following the IC<sub>50</sub> and IC<sub>20</sub> table below. This data was used to identify an appropriate sub-maximal inhibitory concentration of each active compound, to eventually make it possible to identify putative synergistic effects of the mixtures of the active compounds in the presence of retinol. The data in the following table represents the IC<sub>50</sub> and IC<sub>20</sub> (80% of control) values and the concentrations used when testing synergies with combinations of boosters.

In order to demonstrate synergy of two compounds, it is essential to select concentrations to test that are at most IC<sub>20</sub>, in other words, a compound concentration that individually boosts the retinol inhibition of Tgase expression by 20%. Two such compounds should have an additive inhibition of 40%. Using this strategy to determine concentrations leaves a window of 40-100% for further inhibition for detecting synergy of the two compounds under examination.

A more challenging concentration criterion would be selecting concentrations of compounds which alone showed no inhibition effect, but in combination show inhibition. In this study however, we chose an even more challenging criteria. We selected concentrations of compounds that were 10 to 1000 fold lower than the minimally effective Tgase inhibiting concentration. Identification of synergistic combinations using such very low concentrations would mean

that the most effective synergistic combinations were identified.

Booster Class	Compound Name	IC50	IC20	Con. Used for synergy (binary, tertiary, quaternary)
B1	Linoleoyl Monoethanolamide (LAMEA)	1.61E-05	1.48E-05	1E-05 to 1E-09
	Palmitamide Monoethanolamide	ND	ND	1E-06 to 1E-10
	Oleyl Betaine	2.80E-05	1.08E-05	1E-05 to 1E-8
	Naringenin	ND	ND	1E-05 to 1E-09
	Echinacea	ND	ND	1E-05 to 1E-09
	Dimethyl imidazolinone	ND	ND	1E-05 to 1E-09
	Melinamide	ND	ND	1E-05 to 1E-09
	Geranyl geraniol	ND	ND	1E-05 to 1E-09
	Farnesol	9.35E-05	7.82E-05	1E-06 to 1E-09
	Geraniol	7.83E-03	4.72E-03	1E-03 to 1E-07
	$\alpha$ -Damascone	3.35E-04	1.69E-04	1E-04 to 1E-08
	$\alpha$ -Ionone	9.27E-04	1.42E-04	1E-04 to 1E-08
	Castor oil Methyl Ester Acid (MEA)	3.25E-05	9.38E-06	1E-06 to 1E-09
	Ursolic Acid	1.46E-06	5.94E-07	1E-06 to 1E-09
	Utrecht-2	3.47E-06	3.30E-06	1E-06 to 1E-09
	Cocoyl hydroxyethylimidazoline	2.84E-07	9.21E-08	1E-08 to 1E-11
	Acetyl sphingosine (C2 Ceramide)	6.78E-06	5.15E-06	1E-06 to 1E-09
	Hexanoyl sphingosine (C6 Ceramide)	9.99E-05	6.94E-05	1E-05 to 1E-09
	Crocetin	3.75E-05	2.52E-05	1E-05 to 1E-09
	Lyrial	1.27E-04	4.00E-05	1E-05 to 1E-09
	N-Hydroxyethyl-2-hydroxydodecyl amide	3.29E-05	2.40E-05	1E-05 to 1E-09
B2	Phosphatidyl Choline	ND	ND	1E-05 to 1E-09
	Sphingomyelin	ND	ND	1E-05 to 1E-09
	TCC	9.64E-07	6.18E-07	1E-07 to 1E-10
	1,2-di-octanoyl-sn-glycero-3-phosphoethanolamide	ND	ND	1E-05 to 1E-09
B3	Amsacrine-HCl	6.26E-06	3.30E-06	1E-06 to 1E-09
	Carbenoxolone	3.61E-07	2.00E-07	1E-07 to 1E-10
	Glycyrrhetic Acid	8.64E-06	5.96E-06	1E-06 to 1E-09
	Linoleic Acid	1.63E-04	8.95E-05	1E-05 to 1E-09
	Linolenic Acid	1.34E-04	1.21E-04	1E-05 to 1E-09
	Arachidonic Acid (Na <sup>+</sup> salt)	ND	ND	1E-05 to 1E-09
	Myristic Acid	1.72E-05	1.05E-05	1E-05 to 1E-09
	Vanillin	9.70E-03	8.47E-03	1E-03 to 1E-06
	Hexadecanedioic acid	1.30E-04	8.40E-05	1E-05 to 1E-09
B4	12-Hydroxystearic acid	2.91E-05	1.45E-05	1E-05 to 1E-09
	Elaidic acid	6.50E-05	5.88E-05	1E-05 to 1E-09
	Linseed oil	ND	ND	1E-05 to 1E-09
	Isostearic acid	6.88E-05	6.23E-05	1E-05 to 1E-09
	2-Hydroxystearic acid	ND	ND	1E-05 to 1E-09
B5	Climbazole	4.47E-06	2.45E-07	1E-07 to 1E-10

	Clotrimazole	ND	ND	1E-05 to 1E-09
	Miconazole	2.78E-07	8.42E-08	1E-08 to 1E-11
	Coumarin	ND	ND	1E-05 to 1E-09
	Ketoconazole	1.85E-07	5.52E-08	1E-08 to 1E-11
	3,4,-Dihydro-2(1H)- quinolinone (Hydrocarbostyryl)	ND	ND	1E-05 to 1E-09
	2- Hydroxyquinoline (Carbostyryl)	3.64E-04	1.70E-04	1E-04 to 1E-08
	Amino Benzotriazole	ND	ND	1E-05 to 1E-09
	Lauryl hydroxyethylimidazoline	4.67E-07	2.69E-07	1E-07 to 1E-10
	Quercetin	6.29E-05	5.11E-05	1E-05 to 1E-09
	Oleoyl hydroxyethylimidazoline	3.02E-05	5.65E-06	1E-06 to 1E-09
	7H-Benzimidazo[2,1- a]Benz[de]-isoquinolin-7-one	8.59E-07	4.69E-07	1E-07 to 1E-09

ND: Not determined or a clear dose response was not observed. For synergies, a wide range of concentration (4  
5 orders of magnitude 10<sup>-5</sup> to 10<sup>-9</sup>M) was tested.

#### Dose response for boosters class B2 to B4

10 The following tables include the data on the dose response  
of boosters belonging to class B2 to B4. Concentration of  
boosters are given in Molar; mean Tgase' level and Standard  
deviation of 4 replicates is expressed as % of control (0.1%  
DMSO and 10<sup>-7</sup>M retinol). Higher numbers (close to 100 or  
15 above 100) indicate no inhibition of Tgase. The lower the  
number, the more potent the inhibitor is at that  
concentration. The IC50 and IC20 values were calculated  
from this dose response table and expressed in the above  
table.

B2 class boosters:

## Phosphatidyl choline (B2)

Concentration	Tgase levels (Mean)	Tgase (SD)
4.4E-05	90.9	0.01
1.47E-05	120.3	10.6
4.89E-06	70.1	11.4
1.63E-06	98.8	0.00
5.43E-07	86.7	6.19
1.8E-07	75.9	20.5
6.0E-08	87.8	3.9
1.2E-08	159	42.3
2.4E-09	85.5	0.39

5

## Sphingomyelin (B2)

Concentration	Tgase levels (Mean)	Tgase (SD)
3.0E-05	45	3.21
1.0E-05	77.8	25.5
3.33E-06	76.4	7.55
1.1E-06	98.8	0.00
3.73E-07	91.6	14.9
1.23E-07	70.0	3.63
4.10E-08	74.6	4.19
8.2E-08	115.2	1.02
1.65E-09	68.4	2.03
3.29E-10	69.2	2.1

10

## TCC (B2)

Concentration	Tgase levels (Mean)	Tgase (SD)
1.14E-03	36.3	4.6
3.8E-04	3.8	0.96
3.31.23E-04	-3.2	0.91
4.22E-05	-11.2	0
1.41E-06	-.3	4.88
4.69E-07	15.9	3.52
6.26E-08	18.9	3.12
1.25E-08	100.2	23.3
6.9E-09	77.6	21.2
1.0E-09	54.4	11.23

5

## 1,2 dioctanoyl-sn-glycero-3-phosphoethanolamide (B2)

Concentration	Tgase levels (Mean)	Tgase (SD)
1.6E-04	58.1	2.08
5.33E-05	95.4	21.3
1.78E-05	104	4.01
5.93E-06	129	0.0
1.98E-06	110	8.74
6.58E-07	92.8	15.78
2.19E-09	88.6	12.3
4.39E-08	127.3	3.39
8.78E-09	119	21.1
1.79E-9	82	15.6

## B3 Class boosters

## Amscrine B3

5

Concentration	Tgase levels (Mean)	Tgase (SD)
3.0E-05	-10	3.29
1.0E-05	1.8	7.45
3.33E-06	64	4.2
1.1E-06	84	0
3.73E-07	109	6.2
1.23E-07	65	15.8
4.10E-08	110	10.5
8.2E-08	131	27
1.65E-09	113	18
3.29E-10	92	8.9

## Carbenoxolone (B3)

10

Concentration	Tgase levels (Mean)	Tgase (SD)
3.0E-06	-7.1	0
1.0E-06	27.3	1.15
3.33E-07	51.7	0
1.1E-07	158	0
3.73E-08	126	4.67
1.23E-08	81	29
4.10E-09	135	6.88
8.2E-10	112	32
1.65E-10	77.8	10.6
3.29E-11	64	49

## Glyrrheticinic acid (B3)

Concentration	Tgase levels (Mean)	Tgase (SD)
3.0E-04	-0.3	3.9
1.0E-05	0.7	3.55
3.33E-05	2.5	2.1
1.1E-06	96.4	0.00
3.73E-06	120	33.2
1.23E-07	112	38
4.10E-07	93	11
8.2E-08	225	108
1.65E-08	103	11
3.29E-9	100	6.2

5

## Linoleic acid (B3)

Concentration	Tgase levels (Mean)	Tgase (SD)
9.0E-03	-6	3.06
3.0E-03	0.1	2.01
1E-03	-16.4	16.3
1.1E-04	4.4	0
3.73E-04	79.2	0
1.23E-05	62.6	6.2
4.10E-05	76.8	3.69
8.2E-06	146	44.2
1.65E-07	106	20.2
3.29E-07	60.2	2.3

10

## Linolenic acid (B3)

Concentration	Tgase levels (Mean)	Tgase (SD)
9.0E-03	-11	8.7
3.0E-03	-5.7	0.74
1E-03	-7.5	7.8
1.1E-04	-23	0
3.73E-04	68	0.57
1.23E-05	94.9	17.2
4.10E-05	65.9	0.03
8.2E-06	119	1.6
1.65E-07	77	8.5
3.29E-07	98	7.0

5

## Myristic acid (B3)

Concentration	Tgase levels (Mean)	Tgase (SD)
1E-03	-2	4.1
1.1E-04	-8	2.3
3.73E-04	-6	1.16
1.23E-05		
4.10E-05	75.1	1.06
8.2E-06	74.2	10.0
1.65E-07	88.9	8.4
3.29E-07	101	4.47
5.0E-08		
1.1E-08		

10

## Vanillin (B3)

Concentration	Tgase levels (Mean)	Tgase (SD)
1.4E-02	21.5	24.2
4.8E-03	93.8	1.7
1E-03	124	15.6
1.1E-04		
3.73E-04	101	14.3
1.23E-05	82	14.6
4.10E-05	98	2.4
8.2E-06	109	22
1.65E-07	80	4
3.29E-07	93	41

5

## B4 Class boosters

## Hexadecanedioic acid (B4)

10

Concentration	Tgase levels (Mean)	Tgase (SD)
1E-03		
1.1E-04	14.2	2.7
3.73E-04	43.4	8.4
1.23E-05	130	0
4.10E-05	105	14
8.2E-06	114	12
1.65E-07	95	1.9
3.29E-07		
5.0E-08	74	6.7
1.1E-08	70	10.4

## 12-hydroxysteric acid (B4)

Concentration	Tgase levels (Mean)	Tgase (SD)
3.73E-04		
1.23E-05	-5.2	2.3
4.10E-05	32.4	5.3
8.2E-06	97.6	0
1.65E-07	90.2	11
3.29E-07	82	28
5.0E-08	81	3.8
1.1E-08	98	24
2.0E-08	118	28
4.3E-09	71	2.3

5

## Elaidic acid (B4)

Concentration	Tgase levels (Mean)	Tgase (SD)
1E-03	12.8	12.1
1.1E-04	8	0.45
3.73E-04	13.8	1.92
1.23E-05	80.9	0
4.10E-05	58.2	8.8
8.2E-06		
1.65E-07	58	0.13
3.29E-07	69	44
5.0E-08	50.5	3.8
1.1E-08		

10

## Linseed Oil (B4)

Concentration	Tgase levels (Mean)	Tgase (SD)
1E-04	138	15
3.73E-05	145	2.5
1.23E-05	88	12
4.10E-06	113	0
8.2E-06	113	13
1.65E-07	96	18
3.29E-07	106	10
5.0E-08	134	22
1.1E-09	83	13
9.9E-10	73	15

5

## Isosteric acid (B4)

Concentration	Tgase levels (Mean)	Tgase (SD)
1E-03	-8.6	3.4
1.1E-04	1.2	3.0
3.73E-04	-5.3	1.1
1.23E-05	80	00
4.10E-05	67	7.9
8.2E-06	103	12.3
1.65E-07	95	5.5
3.29E-07	123	0.5
5.0E-08	78	12.2
1.1E-08	78	29

10

## 2-hydroxysteric acid (B4)

Concentration	Tgase levels (Mean)	Tgase (SD)
9.1E-04	46.6	6.2
3.73E-04	69.3	8.3
1.23E-04	51	8.8
3.10E-05	96.0	0.0
1.2E-05	105	30
3.65E-06	63	8.0
1.29E-06	80	4.7
2.0E-07	142	34
5.1E-08	64	20
1.0E-08	58	17

5

Synergy of Tgase inhibition with binary combinations of boosters

10 To investigate synergistic inhibition of Tgase expression by combinations of 2 different classes of boosters with retinol, selected combinations of compounds were tested at concentrations given in the above table. The concentrations tested were one log order of magnitude less than the  
15 concentration required for minimal inhibition of Tgase activity (i.e. IC<sub>20</sub>). The compounds were tested alone and in combination and the % inhibition of Tgase is given for each compound and the combination.

20 The following examples give the synergistic combinations in all possible binary combinations (B1/B2; B1/B3, B1/B4; B1/B5; B2/B3, B2/B4; B2/B5; B3/B4, B3/B5; B4/B5). When the % inhibition of the combination is more than the inhibition of each compound added together, it indicates synergy (i.e.  
25 Inhibition by combination is greater than inhibition by

compound 1 + compound 2). All the binary combination examples given in the following table synergistically inhibited Tgase.

Binary combinations	Compound 1	Compound 2	TG as % C Compd 1	TG as % C Compd 2	TG % C Combination
B1/B2	Dimethyl imidazolidinone	Phosphatidylcholine	99	97	84
B1/B2	Alpha-demascone	Phosphatidylcholine	95	97	86
B1/B2	Hexanoyl sphingosine	Phosphatidylcholine	109	97	86
B1/B2	Alpha-ionone	Sphingomyelin	101	98	76
B1/B2	1,2 dioctanoyl-sn-glycero-3-phosphoethanolamide	Phosphatidyl choline	106	98	78
B1/B2	Alpha-demascone	Sphingomyelin	95	84	67
B1/B3	1,2 dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine	123	134	75
B1/B3	1,2 dioctanoyl-sn-glycero-3-phosphoethanolamide	Carbenoxelone	123	164	96
B1/B3	Castor oil MEA	Carbenoxelone	96	164	67
B1/B3	Utrecht-2	Amsacrine	102	98	86
B1/B3	Utrecht-2	Carbenoxelone	102	164	91
B1/B3	Hexanoyl sphingosine	Carbenoxelone	122	164	78
B1/B3	Lyrall	Carbenoxelone	120	164	82
B1/B3	Castor oil MEA	Carbenoxelone	110	164	78
B1/B3	Hexanoyl sphingosine	Amsacrine	122	134	92
B1/B3	Hexanoyl sphingosine	Eliadic acid	122	144	85
B1/B3	Alpha ionone	Amsacrine	101	134	78
B1/B3	1,2 dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrhethinic acid	95	92	69
B1/B4	Naringenin	2- hydroxy steric acid	95	112	78
B1/B4	Hexanoyl sphingosine	2- hydroxy steric acid	99.3	112	77
B1/B4	Lyrall	Hexadecanoic acid	120	95	69
B1/B4	Castor oil MEA	Hexadecanedioic acid	110	125	82
B1/B4	Hexanoyl sphingosine	Isostearic acid	122	146	93
B1/B4	Oleoyl betaine	Hexadecanedioic acid	99.5	125	80
B1/B5	Hexanoyl sphingosine	Cocoyl hydroxyethylimidazolin e	99	102	68
B1/B5	Farnesol	Ketokonazole	98	111	84
B1/B5	Hexanoyl sphingosine	Miconazole	99	101	56
B1/B5	Hexanoyl sphingosine	Ketoconazole	99	99	65
B1/B5	Hexanoyl sphingosine	Lauryl hydroxyethylimidiazoline	99	98	51
B1/B5	Utrecht-2	Amino benzotriazole	122	105	83
B1/B5	Hexanoyl sphingosine	3,4-dihydro-2 quinolinone	122	102	89
B1/B5	Hexanoyl sphingosine	Amino benzotriazole	122	126	85
B1/B5	Castor oil MEA	Lauryl hydroxyethylimidiazoline	110	98	56
B1/B5	Hexanoyl sphingosine	Climbazole	122	98	83
B1/B5	Hexanoyl sphingosine	Miconazole	122	99	78
B1/B5	Hexanoyl sphingosine	Ketoconazole	122	110	90

B1/B5	Oleoyl beatine	ketoconazole	96	116	81
B1/B5	Utrecht-2	Lauryl hydroxyethylimidazoline	122	98	57
B1/B5	Alpha-demascone	Oleoyl hydroxyethylimidazoline	112	73	76
B1/B5	Alpha-ionone	Lauryl hydroxyethylimidazoline	101	98	49
B1/B5	Alpha-ionone	Oleoyl hydroxyethylimidazoline	101	73	75
B2/B3	Phosphatidyl choline	Glycyrrhetic acid	98	92	73
B2/B4	Phosphatidyl choline	2-hydroxy steric acid	98	82	70
B2/B5	Phosphatidyl choline	Climbazole	98	102	82
B2/B5	Phosphatidyl choline	Miconazole	98	111	92
B2/B5	Phosphatidyl choline	Ketoconazole	98	101	89
B2/B5	Phosphatidyl choline	Lauryl hydroxyimidazoline	98	106	82
B3/B4	Amscarine	2-hydroxy steric acid	102	82	75
B3/B4	Myristic acid	2-hydroxy steric acid	110	82	78
B3/B5	Amscarine	Aminobenzotriazole	102	98	84
B3/B5	Amscarine	Dimethyl imidazoline	102	112	94
B3/B5	Myristic acid	Climbazole	110	102	82
B4/B5	Linseed oil	Lauryl hydroxyethyl imidazoline	98	73	57
B4/B5	2-hydroxystearic acid	Ketaconazole	92	109	77
B4/B5	Linseed oil	Oleoyl hydroxyethylimidazoline	98	92	75
B4/B5	2-hydroxystearic acid	Coumarin	92	96	70

### Synergy of Tgase inhibition with tertiary combinations of boosters

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To investigate synergistic inhibition of Tgase expression by combinations of 3 different classes of boosters with retinol, selected combinations of compounds were tested.

10 The concentrations tested were one log order of magnitude less than the concentration required for minimal inhibition of Tgase activity (i.e. IC<sub>20</sub>). The compounds were tested alone and in combination and the % inhibition of Tgase is given for each compound and the combination. The following

15 examples give the synergistic combinations in all possible tertiary combinations (B1/B2/B3; B1/B2/B4; B1/B2/B5;

B1/B3/B4; B1/B3/B5; B1/B4/B5; B2/B3/B4; B2/B3/B5; B2/B4/B5; B3/B4/B5 ). The % inhibition of the combination is more than the inhibition of each compound added together, which indicates synergy (i.e. Inhibition by combination is greater than inhibition by compound 1 + compound 2 + compound 3). All the examples of tertiary combinations of boosters given in the following table synergistically inhibited Tgase in the presence of 10<sup>-7</sup>M retinol.

Compound 1	Compound 2	Compound 3	TG as % C Compd 1	TG as % C Compd 2	TG as % C Compd 3	TG as % C Combo
B1/B2/B3 combinations:						
Phosphatidyl Choline	Glycyrrhetinic Acid	Castor oil Methyl Ester Acid (MEA)	88	91	85	53
Phosphatidyl Choline	Glycyrrhetinic Acid	Echinacea	88	91	119	52
Phosphatidyl Choline	Glycyrrhetinic Acid	Naringenin	88	91	94	52
Phosphatidyl Choline	Glycyrrhetinic Acid	Acetyl sphingosine (C2 Ceramide)	88	91	99	58
Phosphatidyl Choline	Glycyrrhetinic Acid	Farnesol	88	91	118	49
1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetinic Acid	a-Damascone	81	91	89	58
1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Phosphatidyl Choline	Naringenin	81	88	94	66
1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Linoleoyl Monoethanolamide (LAMEA)	81	79	127	60
1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Palmitamide Monoethanolamide	81	79	95	63
1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetinic Acid	a-Damascone	81	91	89	58
1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetinic Acid	Naringenin	81	91	94	75
1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetinic Acid	Echinacea	81	91	119	77
1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetinic Acid	Dimethyl imidazolinone	81	91	87	67
Castor oil Methyl Ester Acid (MEA)	Carbenoxelone	Phosphatidyl Choline	85	95	88	63

B1/B2/B4  
Combinations:

B1/B2/B5

Combinations:

Phosphatidyl Choline	Climbazole	Echinacea	88	84	119	75
Phosphatidyl Choline	Climbazole	Naringenin	88	84	94	83
Phosphatidyl Choline	Climbazole	Geraniol	88	84	105	76
Phosphatidyl Choline	Climbazole	Farnesol	88	84	118	82
Phosphatidyl Choline	Climbazole	Acetyl sphingosine (C2 Ceramide)	88	84	99	82
Phosphatidyl Choline	Miconazole	a-Ionone	88	92	88	70
Phosphatidyl Choline	Miconazole	Castor oil Methyl Ester Acid (MEA)	88	92	85	72

B1/B3/B4

Combinations:

Amsacrine-HCl	Dimethyl imidazolinone	Elaidic acid	79	87	93	0
□-Ionone	Amsacrine-HCl	12-Hydroxystearic acid	68	79	95	62
Lyrial	Hexadecanedioic acid	Vanillin	97	90	134	81
Hexanoyl sphingosine (C6 Ceramide)	Isostearic acid	Glycyrrhetic Acid	104	87	91	58

B1/B3/B5

Combinations:

Amsacrine-HCl	Dimethyl imidazolinone	2-Hydroxyquinoline(Carbostryl)	79	87	95	32
Amsacrine-HCl	Dimethyl imidazolinone	Lauryl hydroxyethylimidazoline	79	87	52	-13
Amsacrine-HCl	Dimethyl imidazolinone	Quercetin	79	87	92	-24
Amsacrine-HCl	Dimethyl imidazolinone	Oleoyl hydroxyethylimidazoline	79	87	76	39
Amsacrine-HCl	Dimethyl imidazolinone	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	79	87	94	32
Amsacrine-HCl	Dimethyl imidazolinone	Coumarin	79	87	80	30
Hexanoyl sphingosine (C6 Ceramide)	Carbenoxolone	Oleoyl hydroxyethylimidazoline	104	88	76	64
Hexanoyl sphingosine (C6 Ceramide)	3,4,-Dihydro-2(1H)-quinolinone (Hydrocarbostryl)	Vanillin	104	90	134	62
Amsacrine-HCl	Amino Benzotriazole	Echinacea	79	105	119	48
Hexanoyl sphingosine (C6 Ceramide)	Amino Benzotriazole	Sphingomyelin	104	105	60	69
Amsacrine-HCl	Amino Benzotriazole	Acetyl sphingosine (C2 Ceramide)	79	105	99	-7
□-Ionone	Amsacrine-HCl	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	68	79	94	54

Utrecht-2	Carbenoxolone	Quercetin	76	88	92	74
Utrecht-2	Carbenoxolone	Oleoyl hydroxyethylimidazole	76	88	76	69
Utrecht-2	Carbenoxolone	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	76	88	94	73
Utrecht-2	Carbenoxolone	3,4,-Dihydro-2(1H)-quinolinone (Hydrocarbostyryl)	76	88	90	70
Myristic Acid	Climbazole	Geraniol	79	84	105	74
Myristic Acid	Climbazole	$\alpha$ -Damascone	79	84	89	73
Myristic Acid	Climbazole	Acetyl sphingosine (C2 Ceramide)	79	84	99	70
Oleyl Betaine	Ketoconazole	Carbenoxolone	62	85	88	78
Oleyl Betaine	Ketoconazole	Glycyrrhetic Acid	62	85	91	71
Oleyl Betaine	Ketoconazole	Linoleic Acid	62	85	11	83
Oleyl Betaine	Ketoconazole	Linolenic Acid	62	85	208	80
Hexanoyl sphingosine (C6 Ceramide)	3,4,-Dihydro-2(1H)-quinolinone (Hydrocarbostyryl)	Vanillin	104	90	134	62
<b>B1/B4/B5</b>						
Combinations:						
Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	Castor oil Methyl Ester Acid (MEA)	93	95	85	75
Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	Naringenin	93	95	94	86
Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	$\alpha$ -Damascone	93	95	89	80
Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	Farnesol	93	95	118	82
Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	Crocin	93	95	90	78
<b>B2/B3/B4</b>						
Combinations:						
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	12-Hydroxystearic acid	81	91	95	57
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Linseed oil	81	91	103	62
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Elaidic acid	81	91	93	75
Phosphatidyl Choline	2-Hydroxystearic acid	Arachidonic Acid (Na <sup>+</sup> salt)	88	83	78	60
<b>B2/B3/B5</b>						
Combinations:						
Phosphatidyl Choline	Climbazole	Linolenic Acid	88	84	208	84
Phosphatidyl Choline	Climbazole	Arachidonic Acid (Na <sup>+</sup> salt)	88	84	78	83
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Climbazole	81	79	84	58
1,2-dioctanoyl-sn-	Amsacrine-HCl	7H-	81	79	94	59

glycero-3-phosphoethanolamide		Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one				
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	3,4,-Dihydro-2(1H)-quinolinone (Hydrocarbostyryl)	81	91	90	56
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	2-Hydroxyquinoline (Carbostyryl)	81	91	95	75
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Amino Benzotriazole	81	91	105	72
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Lauryl hydroxyethylimidazoline	81	91	52	79
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Quercetin	81	91	92	73
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Climbazole	81	91	84	54
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Clotrimazole	81	91	79	42
1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Miconazole	81	91	82	43
<b>B2/B4/B5</b>						
<b>Combinations:</b>						
Phosphatidyl Choline	2-Hydroxystearic acid	Amino Benzotriazole	88	83	105	77
Phosphatidyl Choline	2-Hydroxystearic acid	Lauryl hydroxyethylimidazoline	88	83	52	74
Phosphatidyl Choline	2-Hydroxystearic acid	Quercetin	88	83	92	69
Phosphatidyl Choline	2-Hydroxystearic acid	Oleoyl hydroxyethylimidazoline	88	83	76	75
Phosphatidyl Choline	2-Hydroxystearic acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	88	83	94	79
Phosphatidyl Choline	Climbazole	Elaidic acid	88	84	93	81
<b>B3/B4/B5</b>						
<b>Combinations:</b>						
Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	Carbenoxolone	93	95	88	69
Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	Vanillin	93	95	134	81
Amsacrine-HCl	Amino Benzotriazole	Linseed oil	79	105	103	45
Myristic Acid	Climbazole	12-Hydroxystearic acid	79	84	95	81
Myristic Acid	Climbazole	Linseed oil	79	84	103	81
Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	Arachidonic Acid (Na <sup>+</sup> salt)	93	95	78	63

## 5 Synergy of Tgase inhibition with quaternary combinations of boosters

To investigate synergistic inhibition of Tgase expression by combinations of 4 different classes of boosters with retinol, selected combinations of compounds were tested. The concentrations tested were one log order of magnitude less than the concentration required for minimal inhibition of Tgase activity (i.e. IC<sub>20</sub>).

The compounds were tested alone and in combination and the % inhibition of Tgase is given for each compound and the combination. The following examples give the synergistic combinations in all possible quaternary combinations (B1/B2/B3/B4; B1/B2/B3/B5; B1/B2/B4/B5; B1/B3/B4/B5; B2/B3/B4/B5;). Synergy was confirmed if the difference in % inhibition of the combination (of 4 boosters) is more than 30% that of the inhibition by 3 booster combinations (i.e. % inhibition of 4 booster combo is equal to or greater than % inhibition of 3 booster combo + 30%). All the quaternary combinations of boosters shown in the table given below showed synergy.

20

Compound 1	Compound 2	Compound 3	Compound 4	Quarter-nary TG (%C)	Tertiary (1-3 combo; TG %C)	Difference (<30%=synergy)
<b>B1/B2/B3/B4 Combination:</b>						
Castor oil Methyl Ester Acid (MEA)	Phosphatidyl Choline	Glycyrrhetic Acid	12-Hydroxy-stearic acid	21	64	42
Naringenin	Phosphatidyl Choline	Glycyrrhetic Acid	12-Hydroxy-stearic acid	15	57	41
Linoleoyl Monoethanolamide (LAMEA)	1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	12-Hydroxy-stearic acid	-3	40	43
Linoleoyl Monoethanolamide (LAMEA)	1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Isostearic acid	5	40	35
Linoleoyl Monoethanolamide (LAMEA)	1,2-diocanoyl-sn-glycero-3-phosphoethanol-	Amsacrine-HCl	12-Hydroxy-stearic acid	-3	42	45

Linoleoyl Monoethanolamide (LAMEA)	amide 1,2-dioctanoyl- sn-glycero-3- phosphoethanol- amide	Amsacrine-HCl	Elaidic acid	8	42	34
Hexanoyl sphingosine (C6 Ceramide) Lyrial	TCC	Glycyrrhetic Acid	Isostearic acid	7	54	47
Cocoyl hydroxyethylimid- azoline	TCC	Vanilin	Hexadecan- edioic acid	10	48	38
Cocoyl hydroxyethylimid- azoline	1,2-dioctanoyl- sn-glycero-3- phosphoethanol- amide	Glycyrrhetic Acid	Isostearic acid	0	37	37
Cocoyl hydroxyethylimid- azoline	Phosphatidyl Choline	Arachidonic Acid (Na <sup>+</sup> salt)	2-Hydroxy- stearic acid	-1	37	38
Cocoyl hydroxyethylimid- azoline	1,2-dioctanoyl- sn-glycero-3- phosphoethanol- amide	Glycyrrhetic Acid	Linseed oil	-2	45	47
<b>B1/B2/B3/B5 Combination:</b>						
Castor oil Methyl Ester Acid (MEA)	Phosphatidyl Choline	Glycyrrhetic Acid	Climbazole	20	64	44
Castor oil Methyl Ester Acid (MEA)	Phosphatidyl Choline	Glycyrrhetic Acid	Clotrimazole	26	64	38
Castor oil Methyl Ester Acid (MEA)	Phosphatidyl Choline	Glycyrrhetic Acid	Miconazole	9	64	55
Castor oil Methyl Ester Acid (MEA)	Phosphatidyl Choline	Glycyrrhetic Acid	Ketoconazole	5	64	59
Castor oil Methyl Ester Acid (MEA)	Phosphatidyl Choline	Glycyrrhetic Acid	Lauryl hydroxyethylimidazoline	15	64	49
Castor oil Methyl Ester Acid (MEA)	Phosphatidyl Choline	Glycyrrhetic Acid	Oleoyl hydroxyethylimidazoline	2	64	61
Castor oil Methyl Ester Acid (MEA)	Phosphatidyl Choline	Glycyrrhetic Acid	7H-Benzimidazo[2,1- a]Benz[de]-isoquinolin- 7-one	25	64	39
Echinacea	Phosphatidyl Choline	Glycyrrhetic Acid	12-Hydroxystearic acid	18	62	44
Echinacea	Phosphatidyl Choline	Glycyrrhetic Acid	Climbazole	22	62	40
Echinacea	Phosphatidyl Choline	Glycyrrhetic Acid	Clotrimazole	24	62	38
Echinacea	Phosphatidyl Choline	Glycyrrhetic Acid	Miconazole	13	62	50
Echinacea	Phosphatidyl Choline	Glycyrrhetic Acid	Ketoconazole	12	62	50
Echinacea	Phosphatidyl Choline	Glycyrrhetic Acid	Lauryl hydroxyethylimidazoline	14	62	49
Echinacea	Phosphatidyl Choline	Glycyrrhetic Acid	Oleoyl hydroxyethylimidazoline	3	62	59
Echinacea	Phosphatidyl Choline	Glycyrrhetic Acid	7H-Benzimidazo[2,1- a]Benz[de]-isoquinolin- 7-one	24	62	39
Naringenin	Phosphatidyl Choline	Glycyrrhetic Acid	Miconazole	1	57	56
Naringenin	Phosphatidyl Choline	Glycyrrhetic Acid	Ketoconazole	22	57	34
Naringenin	Phosphatidyl Choline	Glycyrrhetic Acid	Lauryl hydroxyethylimidazoline	10	57	46
Naringenin	Phosphatidyl Choline	Glycyrrhetic Acid	Oleoyl hydroxyethylimidazoline	2	57	54
Naringenin	Phosphatidyl Choline	Glycyrrhetic Acid	7H-Benzimidazo[2,1- a]Benz[de]-isoquinolin-	15	57	42

			7-one			
Palmitamide	Phosphatidyl	Glycyrrhetic	Miconazole	-2	39	41
Monoethanolamide	Choline	Acid				
Palmitamide	Phosphatidyl	Glycyrrhetic	Oleoyl	6	39	33
Monoethanolamide	Choline	Acid	hydroxyethylimidazoline			
Farnesol	Phosphatidyl	Glycyrrhetic	Miconazole	3	43	40
	Choline	Acid				
Farnesol	Phosphatidyl	Glycyrrhetic	Oleoyl	6	43	37
	Choline	Acid	hydroxyethylimidazoline			
Geraniol	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Miconazole	11	47	36
Geraniol	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Oleoyl hydroxyethylimidazoline	3	47	44
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Climbazole	2	40	37
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Miconazole	5	40	35
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Ketoconazole	0	40	40
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Lauryl hydroxyethylimidazoline	-2	40	41
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	Oleoyl hydroxyethylimidazoline	5	40	35
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetic Acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	1	40	39
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Climbazole	7	42	35
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Clotrimazole	10	42	32
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Miconazole	5	42	37
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Ketoconazole	11	42	32
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Lauryl hydroxyethylimidazoline	-4	42	46
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	Oleoyl hydroxyethylimidazoline	5	42	37
Linoleoyl Monoethanolamide (LAMEA)	1,2-dioctanoyl-sn-glycero-3-phosphoethanolamide	Amsacrine-HCl	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	8	42	35

	amide					
Palmitamide	1,2-diocctanoyl-	Amsacrine-HCl	Miconazole	13	43	30
Monoethanolamide	sn-glycero-3-phosphoethanol-					
	amide					
Palmitamide	1,2-diocctanoyl-	Amsacrine-HCl	Oleoyl	3	43	40
Monoethanolamide	sn-glycero-3-phosphoethanol-		hydroxyethylimidazoline			
	amide					
Alpha-Damascone	1,2-diocctanoyl-	Amsacrine-HCl	Miconazole	11	48	37
	sn-glycero-3-phosphoethanol-					
	amide					
Alpha-Damascone	1,2-diocctanoyl-	Amsacrine-HCl	Ketoconazole	13	48	34
	sn-glycero-3-phosphoethanol-					
	amide					
Alpha-Damascone	1,2-diocctanoyl-	Amsacrine-HCl	Lauryl	15	48	33
	sn-glycero-3-phosphoethanol-		hydroxyethylimidazoline			
	amide					
Alpha-Damascone	1,2-diocctanoyl-	Amsacrine-HCl	Oleoyl hydroxyethylimid-	3	48	45
	sn-glycero-3-phosphoethanol-		azoline			
	amide					
Castor oil Methyl	Phosphatidyl	Carbenoxolone	12-Hydroxystearic acid	3	55	52
Ester Acid (MEA)	Choline					
Castor oil Methyl	Phosphatidyl	Carbenoxolone	Climbazole	6	55	49
Ester Acid (MEA)	Choline					
Castor oil Methyl	Phosphatidyl	Carbenoxolone	Miconazole	-2	55	57
Ester Acid (MEA)	Choline					
Castor oil Methyl	Phosphatidyl	Carbenoxolone	Ketoconazole	1	55	54
Ester Acid (MEA)	Choline					
Castor oil Methyl	Phosphatidyl	Carbenoxolone	Lauryl hydroxyethylimi-	4	55	51
Ester Acid (MEA)	Choline		dazoline			
Castor oil Methyl	Phosphatidyl	Carbenoxolone	Oleoyl	3	55	52
Ester Acid (MEA)	Choline		hydroxyethylimidazoline			
Castor oil Methyl	Phosphatidyl	Carbenoxolone	7H-Benzimidazo[2,1-	11	55	44
Ester Acid (MEA)	Choline		a]Benz[de]-isoquinolin-			
			7-one			
Naringenin	Phosphatidyl	Linoleic Acid	Climbazole	-1	45	46
	Choline					
Geraniol	Phosphatidyl	Linoleic Acid	Climbazole	1	40	39
	Choline					
Acetyl sphingosine	Phosphatidyl	Linoleic Acid	Climbazole	0	40	40
(C2 Ceramide)	Choline					
Acetyl sphingosine	Phosphatidyl	Linolenic Acid	Climbazole	10	40	30
(C2 Ceramide)	Choline					
Dimethyl	TCC	Amsacrine-HCl	Elaidic acid	14	47	33
imidazolinone						
Dimethyl	TCC	Amsacrine-HCl	Quercetin	12	44	32
imidazolinone						
Dimethyl	TCC	Amsacrine-HCl	Coumarin	14	58	44
imidazolinone						
Hexanoyl	TCC	Glycyrrheticin	Amino Benzotriazole	8	48	40
sphingosine (C6		Acid				
Ceramide)						
Alpha-Damascone	TCC	Myristic Acid	Climbazole	10	44	34

B1/B2/B4/B5  
Combination:

Lyrial	Vanillin	Hexadecanedioic acid	Miconazole	12	48	36
Lyrial	Vanillin	Hexadecanedioic acid	Oleoyl hydroxyethylimidazoline	4	48	45
Crocetin	TCC	Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	11	48	37
Hexanoyl sphingosine (C6 Ceramide)	Glycyrhethinic Acid	12-Hydroxystearic acid	Amino Benzotriazole	14	48	33
Dimethyl imidazolinone	Phosphatidyl Choline	2-Hydroxystearic acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	2	44	42
Melinamide	Phosphatidyl Choline	2-Hydroxystearic acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	5	44	39
Geranyl geraniol	Phosphatidyl Choline	2-Hydroxystearic acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	9	44	35
Cocoyl hydroxyethylimidazoline	Phosphatidyl Choline	2-Hydroxystearic acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	-8	44	52
Acetyl sphingosine (C2 Ceramide)	Phosphatidyl Choline	2-Hydroxystearic acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	10	44	34
Crocetin	Phosphatidyl Choline	2-Hydroxystearic acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	10	44	34
N,N-Diethyl Cocamide (Cocamide DEA)	Phosphatidyl Choline	2-Hydroxystearic acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	4	44	40
Cocoyl hydroxyethylimidazoline	Phosphatidyl Choline	Elaidic acid	Climbazole	-4	30	34

## B1/B3/B4/B5

## Combination:

Dimethyl imidazolinone	Amsacrine-HCl	Elaidic acid	Miconazole	7	47	40
Dimethyl imidazolinone	Amsacrine-HCl	Elaidic acid	Ketoconazole	6	47	41
Dimethyl imidazolinone	Amsacrine-HCl	Elaidic acid	Oleoyl hydroxyethylimidazoline	3	47	44
Hexanoyl sphingosine (C6 Ceramide)	Glycyrhethinic Acid	Isostearic acid	Clotrimazole	20	54	34
Hexanoyl sphingosine (C6 Ceramide)	Glycyrhethinic Acid	Isostearic acid	Miconazole	10	54	43
Hexanoyl sphingosine (C6 Ceramide)	Glycyrhethinic Acid	Isostearic acid	Lauryl hydroxyethylimidazoline	20	54	33
Hexanoyl sphingosine (C6 Ceramide)	Glycyrhethinic Acid	Isostearic acid	Oleoyl hydroxyethylimidazoline	5	54	48
Crocetin	Linoleic Acid	Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	0	48	48
Crocetin	Linolenic Acid	Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	-2	48	50
Castor oil Methyl Ester Acid (MEA)	Linoleic Acid	Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	-1	31	32
Cocoyl hydroxyethylimidazoline	Carbenoxolone	Elaidic acid	2-Hydroxyquinoline (Carbostyryl)	-6	28	34

## B2/B3/B4/B5

## Combination:

1,2-diocanoyl-sn-glycero-3-phosphoethanol-amide	Glycyrrhetinic Acid	Isostearic acid	Ketoconazole	4	37	33
1,2-diocanoyl-sn-glycero-3-phosphoethanol-amide	Glycyrrhetinic Acid	Isostearic acid	Oleoyl hydroxyethylimidazoline	6	37	31
Phosphatidyl Choline	Arachidonic Acid (Na+ salt)	2-Hydroxystearic acid	Miconazole	6	37	31
Phosphatidyl Choline	Arachidonic Acid (Na+ salt)	2-Hydroxystearic acid	Oleoyl hydroxyethylimidazoline	5	37	32
1,2-diocanoyl-sn-glycero-3-phosphoethanolamide	Glycyrrhetinic Acid	Linseed oil	Miconazole	-1	45	47
1,2-diocanoyl-sn-glycero-3-phosphoethanol-amide	Glycyrrhetinic Acid	Linseed oil	Oleoyl hydroxyethylimidazoline	7	45	38
Phosphatidyl Choline	Carbenoxolone	2-Hydroxystearic acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	8	44	36
Phosphatidyl Choline	Linoleic Acid	2-Hydroxystearic acid	7H-Benzimidazo[2,1-a]Benz[de]-isoquinolin-7-one	-3	44	47
Phosphatidyl Choline	Glycyrrhetinic Acid	Elaidic acid	Climbazole	-3	30	33
Phosphatidyl Choline	Linoleic Acid	Elaidic acid	Climbazole	-2	30	32

Cosmetically Acceptable Vehicle

The composition according to the invention also comprises a  
 5 cosmetically acceptable vehicle to act as a dilutant,  
 dispersant or carrier for the active components in the  
 composition, so as to facilitate their distribution when the  
 composition is applied to the skin.

10 Vehicles other than or in addition to water can include  
 liquid or solid emollients, solvents, humectants, thickeners  
 and powders. An especially preferred non-aqueous carrier is  
 a polydimethyl siloxane and/or a polydimethyl phenyl  
 siloxane. Silicones of this invention may be those with  
 15 viscosities ranging anywhere from about 10 to 10,000,000  
 centistokes at 25°C. Especially desirable are mixtures of low

and high viscosity silicones. These silicones are available from the General Electric Company under trademarks Vicasil, SE and SF and from the Dow Corning Company under the 200 and 550 Series. Amounts of silicone which can be utilised in the compositions of this invention range anywhere from 5 to 95%, preferably from 25 to 90% by weight of the composition.

#### Optional Skin Benefit Materials and Cosmetic Adjuncts

10 An oil or oily material may be present, together with an emulsifier to provide either a water-in-oil emulsion or an oil-in-water emulsion, depending largely on the average hydrophilic-lipophilic balance (HLB) of the emulsifier employed.

15 Various types of active ingredients may be present in cosmetic compositions of the present invention. Various types of active ingredients may be present in cosmetic compositions of the present invention. Actives are defined as skin or hair benefit agents other than emollients and other than ingredients that merely improve the physical characteristics of the composition. Although not limited to this category, general examples include sunscreens, skin lightening agents, and tanning agents.

25 Sunscreens include those materials commonly employed to block ultraviolet light. Illustrative compounds are the derivatives of PABA, cinnamate and salicylate. For example, octyl methoxycinnamate and 2-hydroxy-4-methoxy benzophenone (also known as oxybenzone) can be used. Octyl

30

methoxycinnamate and 2-hydroxy-4-methoxy benzophenone are commercially available under the trademarks, Parsol MCX and Benzophenone-3, respectively.

- 5 The exact amount of sunscreen employed in the emulsions can vary depending upon the degree of protection desired from the sun's UV radiation.

Another preferred optional ingredient is selected from  
10 essential fatty acids (EFAs), i.e., those fatty acids which are essential for the plasma membrane formation of all cells, in keratinocytes EFA deficiency makes cells hyperproliferative. Supplementation of EFA corrects this. EFA's also enhance lipid biosynthesis of epidermis and  
15 provide lipids for the barrier formation of the epidermis. The essential fatty acids are preferably chosen from linoleic acid,  $\gamma$ -linolenic acid, homo-  $\gamma$ -linolenic acid, columbinic acid, eicosa-(n-6,9,13)-trienoic acid, arachidonic acid,  $\gamma$ -linolenic acid, timnodonic acid, hexaenoic acid and mixtures  
20 thereof.

Emollients are often incorporated into cosmetic compositions of the present invention. Levels of such emollients may range from about 0.5% to about 50%, preferably between about  
25 5% and 30% by weight of the total composition. Emollients may be classified under such general chemical categories as esters, fatty acids and alcohols, polyols and hydrocarbons.

Esters may be mono- or di-esters. Acceptable examples of  
30 fatty di-esters include dibutyl adipate, diethyl sebacate,

diisopropyl dimerate, and dioctyl succinate. Acceptable branched chain fatty esters include 2-ethyl-hexyl myristate, isopropyl stearate and isostearyl palmitate. Acceptable tribasic acid esters include triisopropyl trilinoleate and  
5 trilauryl citrate. Acceptable straight chain fatty esters include lauryl palmitate, myristyl lactate, oleyl eurate and stearyl oleate. Preferred esters include coco-caprylate/caprate (a blend of coco-caprylate and coco-caprate), propylene glycol myristyl ether acetate,  
10 diisopropyl adipate and cetyl octanoate.

Suitable fatty alcohols and acids include those compounds having from 10 to 20 carbon atoms. Especially preferred are such compounds such as cetyl, myristyl, palmitic and stearyl  
15 alcohols and acids.

Among the polyols which may serve as emollients are linear and branched chain alkyl polyhydroxyl compounds. For example, propylene glycol, sorbitol and glycerin are  
20 preferred. Also useful may be polymeric polyols such as polypropylene glycol and polyethylene glycol. Butylene and propylene glycol are also especially preferred as penetration enhancers.

25 Exemplary hydrocarbons which may serve as emollients are those having hydrocarbon chains anywhere from 12 to 30 carbon atoms. Specific examples include mineral oil, petroleum jelly, squalene and isoparaffins.

Another category of functional ingredients within the cosmetic compositions of the present invention are thickeners. A thickener will usually be present in amounts anywhere from 0.1 to 20% by weight, preferably from about 5 0.5% to 10% by weight of the composition. Exemplary thickeners are cross-linked polyacrylate materials available under the trademark Carbopol from the B.F. Goodrich Company. Gums may be employed such as xanthan, carrageenan, gelatin, karaya, pectin and locust beans gum. Under certain 10 circumstances the thickening function may be accomplished by a material also serving as a silicone or emollient. For instance, silicone gums in excess of 10 centistokes and esters such as glycerol stearate have dual functionality.

15 Powders may be incorporated into the cosmetic composition of the invention. These powders include chalk, talc, Fullers earth, kaolin, starch, smectite clays, chemically modified magnesium aluminum silicate, organically modified montmorillonite clay, hydrated aluminum silicate, fumed 20 silica, aluminum starch octenyl succinate and mixtures thereof.

Other adjunct minor components may also be incorporated into the cosmetic compositions. These ingredients may include 25 coloring agents, opacifiers and perfumes. Amounts of these materials may range anywhere from 0.001% up to 20% by weight of the composition.

### Use of the Composition

The composition according to the invention is intended primarily as a product for topical application to human skin, especially as an agent for conditioning and smoothening the skin, and preventing or reducing the appearance of wrinkled or aged skin.

In use, a small quantity of the composition, for example from 1 to 5ml, is applied to exposed areas of the skin, from a suitable container or applicator and, if necessary, it is then spread over and/or rubbed into the skin using the hand or fingers or a suitable device.

### Product Form and Packaging

The topical skin treatment composition of the invention can be formulated as a lotion, a fluid cream, a cream or a gel. The composition can be packaged in a suitable container to suit its viscosity and intended use by the consumer. For example, a lotion or fluid cream can be packaged in a bottle or a roll-ball applicator, or a capsule, or a propellant-driven aerosol device or a container fitted with a pump suitable for finger operation. When the composition is a cream, it can simply be stored in a non-deformable bottle or squeeze container, such as a tube or a lidded jar.

The invention accordingly also provides a closed container containing a cosmetically acceptable composition as herein defined.

CLAIMS

1. A skin care composition comprising:
  - a. from 0.001% to 10% of a retinoid;
  - 5    b. a combination of at least 2 retinoid boosters belonging to classes B1 to B5 in an amount of from 0.0001% to 50% where the ratios of the two boosters to each other in the range of is 1:1000 to 1000:1;
  - c. a cosmetically acceptable vehicle.
- 10    2. The skin care composition of claim 1 where the combination of boosters comprises at least three boosters belonging to the classes B1 to B5 in an amount of from 0.0001% to 50%.
- 15    3. The skin care composition of claim 1 or claim 2 where the second composition has a combination of at least 4 boosters belonging to the classes B1 to B5 in an amount of from 0.0001% to 50%.
- 20    4. The skin care composition of any of the preceding claims where the second composition has a combination of all the 5 classes of boosters belonging to the classes B1 to B5.
- 25    5. A cosmetic method of conditioning skin, the method comprising applying topically to the skin the product of any one of claims 1 through to 5.
- 30    6. A cosmetic method of mimicking the effect on skin or retinoic acid, the method comprising applying to the skin the product of any one of claims 1-5.

7. A skin care composition comprising:
- a. a combination of at least 2 retinoid boosters belonging to classes B1 to B5 in an amount of from 0.0001% to 50%  
5 where the ratios of the two boosters to each other in the range of is 1:1000 to 1000:1;
  - b. a cosmetically acceptable vehicle.

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(54) Title: **SKIN CONDITIONING COMPOSITIONS CONTAINING COMPOUNDS FOR MIMICKING THE EFFECT ON SKIN OF RETINOIC ACID**

(57) Abstract: A skin care product comprising from about 0.001 % to about 10 % of a retinoid, in combination with 0.0001 % to about 50 % of a combination of retinoid boosters.



**WO 02/002074 A3**

## INTERNATIONAL SEARCH REPORT

International Application No.

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A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 A61K7/48

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 803 248 A (UNILEVER PLC ;UNILEVER NV (NL)) 29 October 1997 (1997-10-29) claims 1-9; examples 5-10 & US 5 716 627 A 10 February 1998 (1998-02-10) cited in the application ---	1-7
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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be of particular relevance

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\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*G\* document member of the same patent family

Date of the actual completion of the international search

6 December 2002

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/07234

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 759 556 A (RAWLINGS ANTHONY VINCENT ET AL) 2 June 1998 (1998-06-02) cited in the application example 10 ----	1-7
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 01/07234

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 01 07234

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-7 relate to an extremely large number of possible compositions. In fact, the claims contain so many possible combinations of structurally unrelated compounds that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for the general idea underlying the application only.

Claims searched completely : none

Claims searched uncompletely: 1-7

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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Information on patent family members

International Application No

PCT/EP 01/07234

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